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The Insulin Receptor and Its Substrate: Molecular Determinants of Early Events in Insulin Action

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Insulin is a potent metabolic and growth-promoting hormone that has pleiotropic effects at the level of the cell and within the intact organism. Insulin acts on cells to stimulate glucose, protein, and lipid metabolism, as well as RNA and DNA synthesis, by modifying the activity of a variety of enzymes and transport processes. The glucoregulatory effects of insulin at a whole body level are predominantly exerted by insulin action on liver, fat, and muscle. In liver, insulin stimulates glucose incorporation into glycogen and inhibits the production of glucose by glycogenolysis and gluconeogenesis. In muscle and fat, insulin stimulates glucose uptake, storage, and metabolism. In addition to these more classical effects, insulin also stimulates glucose metabolism in many other tissues that play little or no role in overall glucose homeostasis. In these nonclassical target tissues, insulin also often acts as a growth factor and in some manner modifies or augments the function of other regulators of metabolism of these cells.

Since the discovery of insulin over 70 years ago, considerable research has been devoted to attempting to understand the molecular mechanism of insulin action. The importance of understanding insulin action has been pointed out by its complex physiologic effects, as well as by the fact that altered insulin action, i.e., insulin resistance, plays important roles in the pathogenesis of many disorders, including obesity, diabetes mellitus, hypertension, and the glucose intolerance associated with many endocrine diseases (Caro *et al.*, 1986, 1987; DeFronzo, 1988; Reaven, 1988; Reddy and Kahn, 1988; Moller and Flier, 1991). It is only with the recent characterization of the insulin receptor as a tyrosine kinase (Kasuga *et al.*, 1982b; Shia and Pilch, 1983; Petruzzelli *et al.*, 1984) and identification of some of

the related, downstream early components of the insulin response system (White *et al.*, 1985; Sun *et al.*, 1991) that we have come to have some understanding of the mechanism of insulin action at a molecular level. In this review, we will focus our attention on the insulin receptor and its primary cellular substrate, IRS-1, as the initial components of this insulin action cascade. We will consider the structure and function of these proteins, the potential mechanisms by which they may be joined to the enzymes that regulate cellular metabolism, as well as how they may communicate with the actions of insulin at the level of the cell nucleus on RNA and DNA.

I. The Insulin Receptor

The actions of insulin at the cellular level are initiated by insulin binding to its plasma membrane receptor. This receptor is present on almost all mammalian cell types, ranging in number from <100 receptors per cell on circulating erthrocytes to >300,000 receptors per cell on hepatocytes and adipocytes (Ginsberg, 1977). In general, the concentration of the receptors is higher in classical target tissues, although there are exceptions to this rule in that muscle tends to have a relatively low concentration of insulin receptors (Caro *et al.*, 1987). In cell types with high receptor content, such as liver and adipose cells, classical metabolic effects occur with only a small fraction of receptors occupied, i.e., there are "spare receptors" for insulin action (Kahn *et al.*, 1981; Gammeltoft, 1984). There is no evidence, however, that this is due to two structural classes of insulin receptors (one of which is active and the other inactive); rather, the high concentration of receptors on cells serves as a driving force for more rapid kinetics of binding for insulin, because the circulating concentration of insulin (10^{-10} to 10^{-9}) is lower than the average affinity for binding (Gammeltoft, 1984).

A. INSULIN RECEPTOR STRUCTURE

The insulin receptor is a heterotetrameric protein consisting of two α subunits and two β subunits linked together by disulfide bonds to form a $\beta\alpha\alpha\beta$ structure (Fig. 1) (Czech, 1985, 1989; Avruch, 1989). The α subunits have an apparent molecular weight (M_r) of 135,000 and the β subunits have an apparent M_r of 95,000. Thus, the calculated molecular weight of the holoreceptor is 460,000, although on SDS–gel electrophoresis under nonreducing conditions the usual estimated size is ~350,000 (Massague *et al.*, 1981; Kasuga *et al.*, 1982a). Both are glycosylated with complex N-linked carbohydrates on the α subunit and with both N- and O-linked carbohydrates on the β subunit (Edge *et al.*, 1990). The role for the

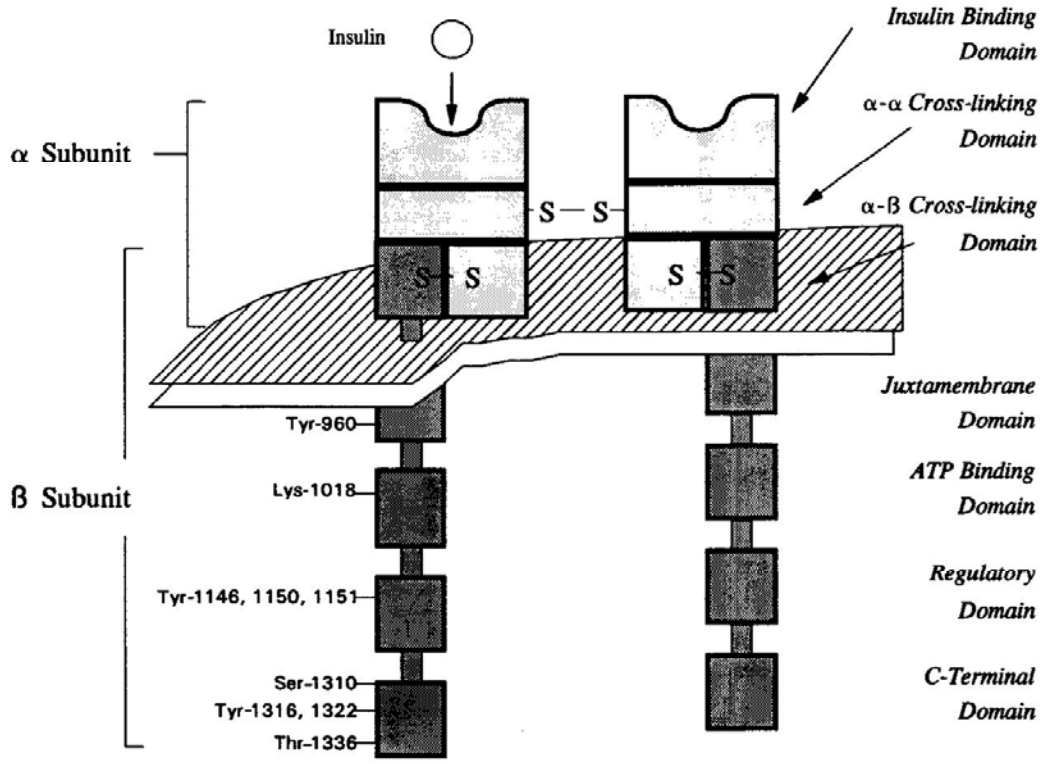

FIG. 1. A schematic model of the insulin receptor in its plasma membrane. The numbering system used in the figure represents that of the − exon 11 sequence. For the + exon 11 sequence, add 12 to each.

glycosylation of the insulin receptor is unknown. The α subunits of insulin receptors in brain and some neural tissues have much lower apparent molecular weights due to differences in glycosylation (Heidenreich *et al.*, 1988).

The two subunits of the insulin receptor are specialized to perform the two functions of this protein. The α subunit is entirely extracellular and contains the insulin binding site (Yip, 1992). The β subunit is a transmembrane protein and contains the tyrosine kinase signaling domain on its intracellular segment (White and Kahn, 1986). The insulin receptor is now recognized to be a member of the family of tyrosine protein kinase receptors for peptide hormones and growth factors. Based on overall structure, three classes of tyrosine kinase receptors have been identified (Yarden and Ullrich, 1988). One class represents monomeric proteins consisting of a single transmembrane polypeptide chain with a single continuous intracellular tyrosine kinase domain, as exemplified by the EGF receptor. A second class, similar to class I but with a discontinuous tyrosine kinase domain, is exemplified by the PDGF and CSF-1 receptors. The insulin receptor and the highly homologous IGF-1 receptor constitute class III.

B. INSULIN RECEPTOR GENE AND RECEPTOR BIOSYNTHESIS

Although the insulin receptor is a tetrameric protein, the receptor is the product of a single gene located on the short arm of chromosome 19 (19p7) near the gene for the LDL receptor (Yang-Feng *et al.*, 1985). The structure of this gene has been elucidated by both restriction mapping (Muller-Wieland *et al.*, 1989) and genomic cloning (Seino *et al.*, 1989, 1990). The insulin receptor gene is about 150 kb in length and contains 22 exons separated by 21 introns (Fig. 2). Exons 1 through 11 are spread over 90 kb and code for the α subunit; exons 12 through 22 occur over a span of 30 kb and code for the β subunit. Although the coding information is contained in about 4 kb of DNA, most cells possess four species of receptor mRNA ranging in length from 5.7 to 9.5 kb (Goldstein *et al.*, 1987; Goldstein and Kahn, 1989). Using RNase mapping, we have shown that the variability in length is primarily due to differences in the 3′-untranslated region with a relatively constant 5′-untranslated domain. Although differences in the 3′-untranslated length have been suggested to contribute to alterations in mRNA half-life, the $t_{1/2}$ for all species of insulin receptor mRNA is about 70 minutes. The concentration of receptor mRNA is greatest in brain and kidney, but is widely expressed in virtually all tissues.

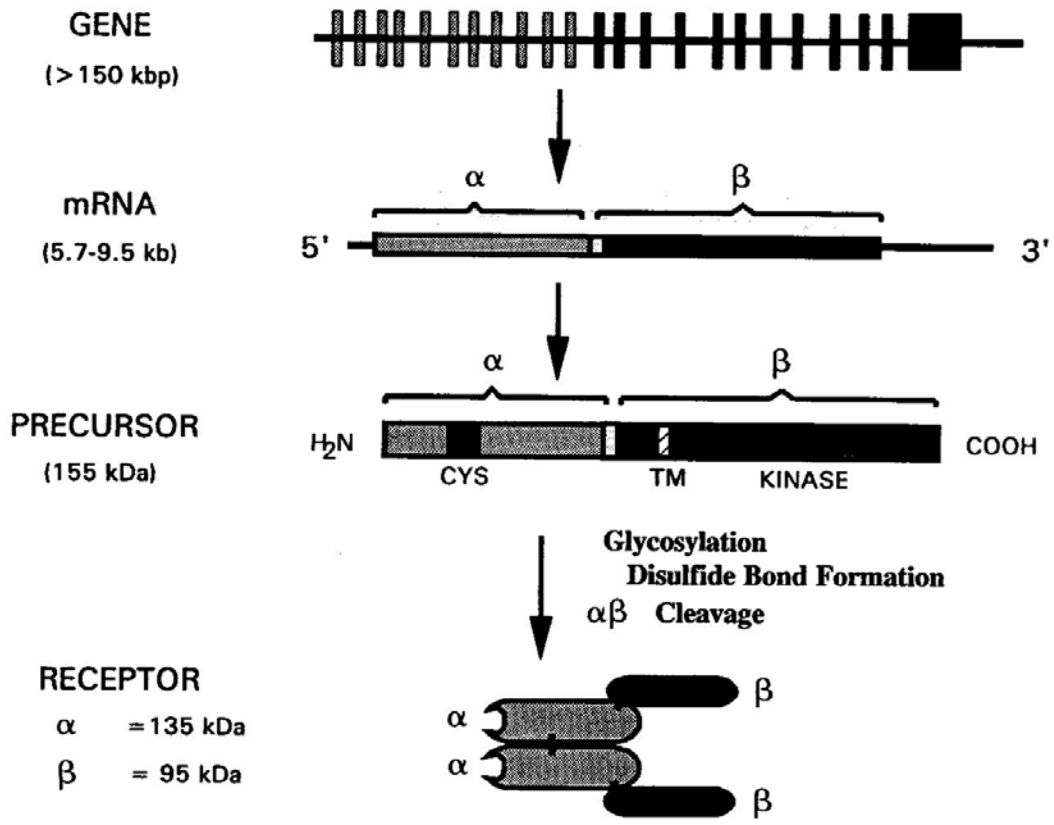

FIG. 2. Insulin receptor biosynthesis.

The insulin receptor mRNAs code for a single-chain precursor of the insulin receptor that contains the entire coding sequence for both the α and β subunits joined by a 4-amino acid connecting peptide (Fig. 2). Two forms of insulin proreceptor that differ in length by 12 amino acids near the C terminus of the α subunit are synthesized from these RNA species (Seino and Bell, 1989; Moller *et al.*, 1989). This is based on alternative splicing of the 36-base pair (bp) exon 11 in the insulin receptor gene producing a larger form of insulin proreceptor containing 1382 amino acids and a smaller form of 1370 amino acids. These were recognized initially by differences in the cDNA cloning (Ullrich *et al.*, 1985; Ebina *et al.*, 1985). Both forms of the insulin receptor (+ and − exon 11) are expressed in most tissues, but the amount varies from tissue to tissue (Table I) (Seino and Bell, 1989; Goldstein and Dudley, 1990). The possible functional significance of these alternatively spliced forms is uncertain, but it appears that the − exon 11 form has a relatively higher affinity for both insulin and especially IGF-1 (Mosthaf *et al.*, 1990; McClain, 1991; Moller *et al.*, 1989; Benecke *et al.*, 1992). In addition, this form is more rapidly internalized and down-regulated (Vogt *et al.*, 1991; Yamaguchi *et al.*, 1991). Mosthaf *et al.* (1991) have also suggested that more of the + exon 11 form is expressed in muscle of patients with non-insulin-dependent diabetes mellitus (NIDDM) as compared to normal individuals and thus may be a marker or may contribute to insulin resistance, but this finding has not been reproduced in other laboratories (Benecke *et al.*, 1992) and thus remains controversial.

After synthesis in the rough endoplasmic reticulum the proreceptor is transported through the golgi apparatus, where it is rapidly glycosylated to give species of 180–210 kDa (Hedo *et al.*, 1981; Hedo and Gorden 1985; Olson *et al.*, 1988). The proreceptors are then disulfide linked, cleaved into α and β subunits, and further glycosylated to give the final active

TABLE I
Tissue Distribution of Insulin Receptor mRNAs[a]

Tissue	Relative concentration	+ Exon 11 (%)
Brain	78	0
Kidney	50	60
Liver	30	90
Spleen	22	0
Placenta	n.d.	50

[a] Tissue distribution of insulin receptor mRNA and relative concentration of + exon 11 variant; n.d., not determined. Adapted from Araki *et al.* (1992), Seino and Bell (1989), and Moller *et al.* (1989).

tetrameric receptor. The proreceptor contains a 27-amino acid signal peptide that helps guide its movement and processing in the cell. A small fraction of the fully glycosylated proreceptor may escape processing and appear on the cell surface; however, this unprocessed receptor form has low affinity for insulin and markedly reduced kinase activity (Williams *et al.*, 1990).

C. DOMAIN STRUCTURE OF THE INSULIN RECEPTOR α SUBUNIT

As noted above, the two subunits of the insulin receptor are specialized to the two functions that this receptor must achieve. The α subunit is specialized for high-affinity insulin binding, and the β subunit is specialized for transmembrane signal transduction. Each of the subunits can also be subdivided into a number of specific functional domains, which in many cases appear to follow the exonic structure of the receptor gene (Seino *et al.*, 1990).

The extracellular α subunit consists of an N-terminal domain (coded for by exons 1–2), a cysteine-rich domain (coded for by exons 3–5), a domain involved in α–α disulfide bonding (of uncertain location), and a domain involved in α–β disulfide bonding (probably coded for by exon 10) (Fig. 1). Based on chemical modification and *in vitro* mutagenesis studies, the most critical domain for high-affinity insulin binding is that coded for by exons 1, 2, and 3 and consists of the first 298 amino acids of the receptor (Yip, 1992; DeMeyts *et al.*, 1990; Rafaeloff *et al.*, 1989). By replacing exons 1–3 of the insulin receptor with the corresponding sequences from the cDNA of the IGF-1 receptor, insulin binding affinity is significantly impaired, and the newly created chimeric receptor binds IGF-1 with high affinity (Andersen *et al.*, 1990; Gustafson and Rutter, 1990; Kjeldsen *et al.*, 1991). Within this region several critical residues have been identified, particularly Phe-89 (DeMeyts *et al.*, 1990). Mutation of this single residue to almost any other amino acid significantly reduces insulin binding to its receptor. If a chimeric receptor is prepared, replacing only exon 3 (corresponding approximately to amino acids 191 to 298) of the insulin receptor with that of the IGF-1 receptor, the resulting chimeric receptor retains high-affinity insulin binding and acquires high-affinity IGF-1 binding (Andersen *et al.*, 1990). These results suggest that these three N-terminal exons contain both positive and negative recognition elements, and that the regions coded for by exons 1 and 2 are most important for insulin recognition, whereas that for exon 3 may confer IGF-1 specificity to the insulin receptor.

Similar conditions have been reached by studies in which the binding domain has been mapped using insulin covalently cross-linked to the

receptor (Yip *et al.*, 1988; Waugh *et al.*, 1989; Wedekind *et al.*, 1989). It is likely, however, that the completely normal high-affinity binding involves a number of conformational determinants contributed by the entire α subunit, and perhaps even the β subunit. Thus, when a solubilized form of receptor ectodomain, including the entire α and extracellular β linked in tetrameric form, is expressed, it binds insulin with significantly lower affinity than the normal intact receptor (Schaefer *et al.*, 1990; Paul *et al.*, 1990). Free α subunits, as well as many receptors containing single point mutations, spread throughout the α subunit, bind insulin very poorly, and most truncated α subunits do not bind insulin at all (Taylor *et al.*, 1990a; Schaefer *et al.*, 1990). As noted above, near the C terminus of the α subunit is a site of alternative mRNA splicing that results in the addition or deletion of 12 amino acids to the sequence. This addition of 12 amino acids has effects on the affinity of the insulin receptor for both insulin and IGF-1, suggesting that in the three-dimensional structure of the receptor, the C-terminal domain of the α subunit may come into juxtaposition to the N-terminal regions, also of the α subunit involved in high-affinity insulin binding. Evidence for such a complex interaction of a peptide hormone with its receptor has recently been obtained for the human growth hormone receptor (see article by Wells *et al.*, 1993). This type of three-dimensional analysis for the insulin receptor will probably require an X-ray crystallographic analysis of the receptor and its subunits.

The intact insulin receptor, both in its solubilized, purified form and in its normal plasma membrane environment, exhibits negative cooperativity between insulin binding sites and curvilinear Scatchard plots (DeMeyts *et al.*, 1976). This means that the insulin receptor binds the first insulin molecule with high affinity, and binds the second insulin molecule with much lower affinity. The molecular mechanism underlying this phenomenon remains uncertain. Although the intact insulin receptor possesses two α subunits, some studies have suggested that only a single molecule of insulin binds with high affinity (Pang and Shafer, 1984; Sweet *et al.*, 1987). However, this property still persists in single tetrameric receptors and in isolated receptors reconstituted in liposomes such that there is no more than one receptor per liposome (Huertas and Kahn, 1992). In our hands, the stoichiometry of binding also suggests that a single tetrameric receptor can bind more than one insulin molecule. Isolated $\alpha\beta$ heterodimers obtained by mild reduction display linear Scatchard plots with a binding affinity intermediate between the high- and low-affinity states of the tetrameric receptor (Sweet *et al.*, 1987; Boni-Schnetzler *et al.*, 1987). Thus, negative cooperativity appears to be the property of a single tetrameric receptor, and is likely due to interactions and three-dimensional conformational changes within the receptor itself (Gu *et al.*, 1988). Changes in the negative cooperative properties have been observed with both natural

mutations and *in vitro* mutations involving residues 460, 647, and the region 682–685 (Kadowaki *et al.*, 1990; Cheatham and Kahn, 1992).

All of the 36 cysteine residues in the α subunit are involved in either disulfide bonding or are otherwise covalently modified and thus contribute to important conformational features of the receptor molecule (Finn *et al.*, 1990). Treatment of the insulin receptor with increasing concentrations of reducing agent results initially in an increase in high-affinity binding, followed by a decrease in binding (Crettaz *et al.*, 1984). The exact residues involved in disulfide bonding between the two α subunits have not yet been identified, but these residues are more sensitive to reductant than those involved in α–β disulfide bonding (Czech, 1985). Although α–α disulfide bonding occurs early in the course of proreceptor synthesis, when the receptor is in its intracellular (and insulin-free) environment (Olson *et al.*, 1988), when the receptor is split into its two half-receptors (or protomers) by mild reduction *in vitro,* insulin addition to the medium provides a driving force for reassociation of the protomers (Boni-Schnetzler *et al.*, 1988). The normal insulin receptor protomer may also form hybrids with the IGF-1 receptor or with mutant insulin receptors, a phenomenon that also occurs both *in vitro* and normally in the intact cell (Treadway *et al.*, 1989, 1991). Although the exact physiological significance of this is uncertain, it has been speculated that this phenomenon may account for some of the growth-promoting actions of insulin and may account for the "dominant-negative" inhibition of normal receptor function when cells also express mutant insulin receptors (Whittaker *et al.*, 1990).

Based on structural analysis of the insulin receptor subjected to mild tryptic proteolysis, the domain of the receptor involved in α–β disulfide bonding included the C-terminal 25 kDa of the α subunit (Shoelson *et al.*, 1988; Xu *et al.*, 1990). This fragment contains only four cysteine residues (the extracellular β subunit also contains four) at positions 647, 682, 683, and 685. To determine which of these Cys residues might be involved in α–β bonding, we prepared and expressed two *in vitro* mutant receptors: one in which Cys-647 was converted to serine, and another in which Cys residues 682, 683, and 685 were converted to serine (Cheatham and Kahn, 1992). The triple mutant exhibited normal insulin binding and receptor structure. By contrast, the Cys-647 mutant exhibited an altered tetrameric structure, with most surface receptors consisting of an α_2 structure noncovalently linked to the two β subunits (Fig. 3). Interestingly, these receptor tetramers remained together on the surface of the cell, but could be dissociated with treatment by urea or acid pH. This noncovalent receptor structure was associated with an increase in receptor affinity and negative cooperativity. This noncovalently associated receptor was defective for insulin-stimulated receptor kinase activation, suggesting that normal disul-

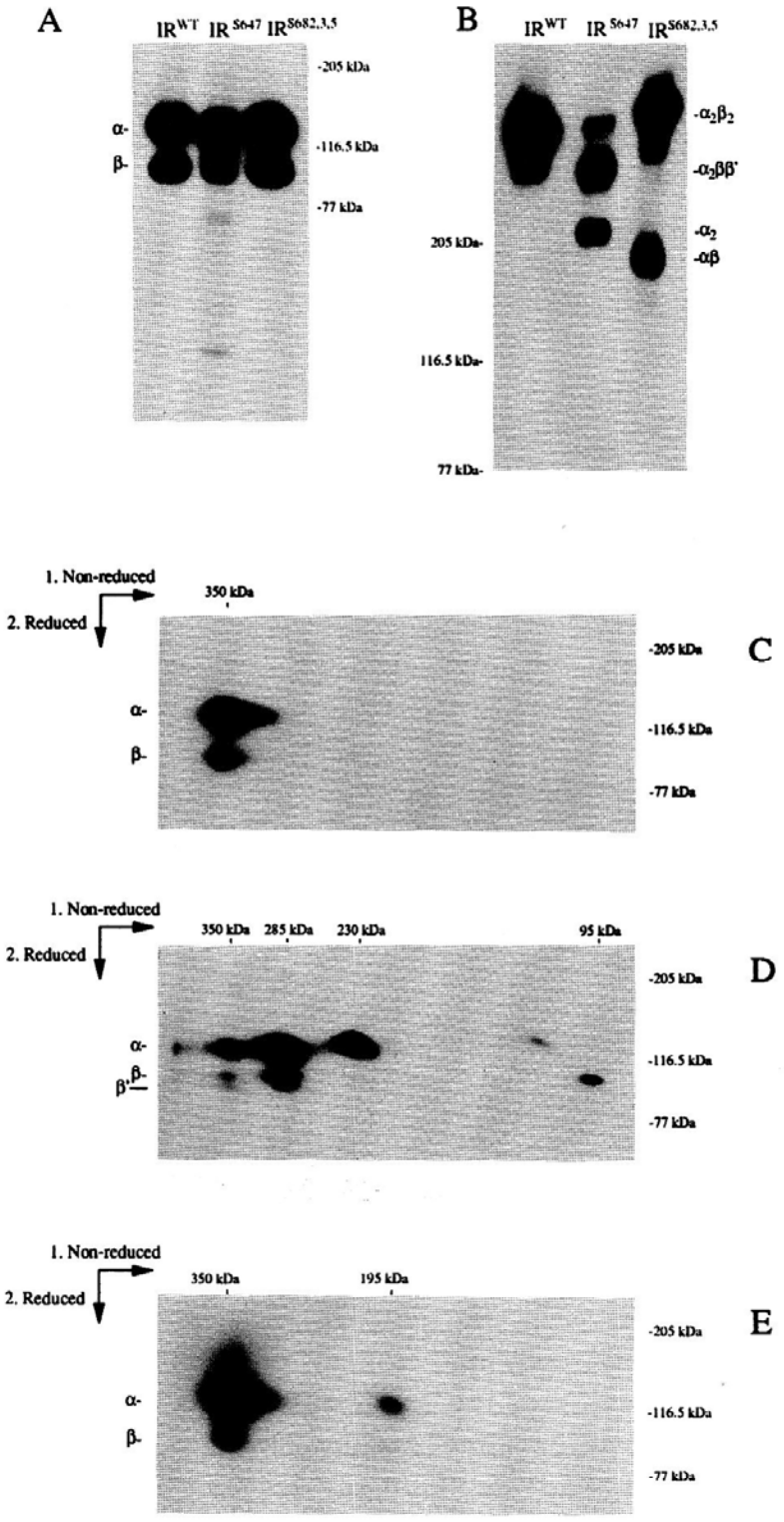

FIG. 3. Structure and function of the insulin receptor with a mutation of Cys-647 (IR^{S647}) and Cys-682, Cys-683, and Cys-685 ($IR^{S682,3,5}$). Left panel: CHO cells overexpressing wild-type (IR^{WT}) or mutant insulin receptors were surface labeled with Na[^{125}I]. Insulin receptors were immunoprecipitated and subjected to SDS–PAGE under reducing (A) or nonreducing (B) conditions. In C–E, receptors from B were subjected to a second electrophoresis under reducing conditions to separate individual components of the oligomeric forms. (C) IR^{WT}; (D) IR^{S647}; (E) $IR^{S682,3,5}$. Right panel: insulin-stimulated receptor autophosphorylation in intact cells following ^{32}P labeling. Receptors were immunoprecipitated with antireceptor antibody. Adapted from Cheatham and Kahn (1992).

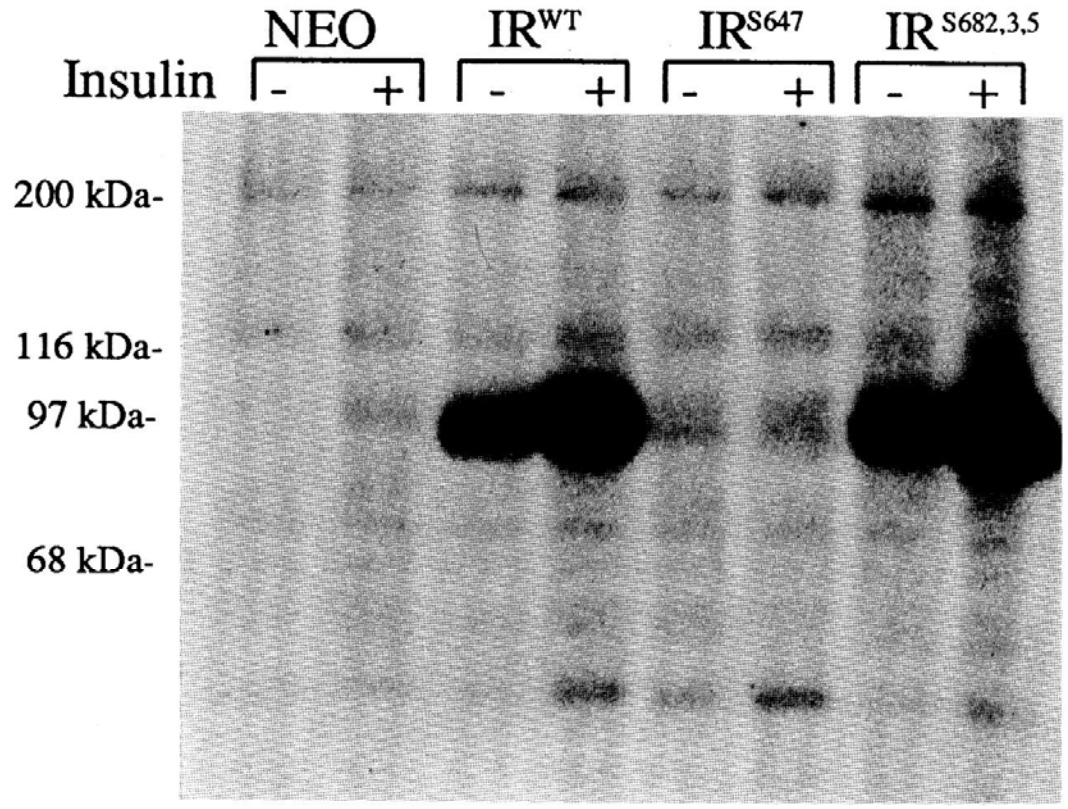

FIG. 3.—*Continued*

fide bond formation between α and β subunits is required for normal signal transduction (Fig. 3).

D. DOMAIN STRUCTURE OF THE INSULIN RECEPTOR β SUBUNIT

The β subunit of the insulin receptor contains an extracellular domain of about 193 amino acids, a single transmembrane domain of 23 amino acids, and a complex intracellular domain of 402 amino acids. Little is known about the function of the extracellular domain of the β subunit except that it contains the cysteine residues involved in interchain bonding between α and β subunits (Cheatham and Kahn, 1992) and sites of both complex N-linked and O-linked glycosylation (Edge *et al.*, 1990). Of the four cysteine residues in the extracellular domain, it is not clear which are involved in the interchain disulfide with Cys-647 of the α subunit.

The transmembrane domain of the β subunit is a single α-helical segment of 23 amino acids that links the extracellular and intracellular domains of the receptor. Because this single α-helical domain forms the only physical link between the extracellular and intracellular β subunits, it should obvi-

ously play a critical role in signal transduction. In general, however, the transmembrane domains of tyrosine kinase receptors show little in the way of conservation of specific sequence, and a number of *in vitro* mutations may be made in this domain, including chimeric receptors, without affecting receptor function (Yarden and Ullrich, 1988; Riedel *et al.*, 1986; Frattali *et al.*, 1991). However, in the case of the insulin receptor, two types of studies suggest that the structure of this domain and its internalization with surrounding lipids may play an important role in transduction of the signal across the lipid bilayer, as well as the cellular trafficking of the receptor. These studies include reconstitution of receptors into liposomes of varying lipid composition and *in vitro* mutagenesis of the transmembrane domain itself.

E. BEHAVIOR OF THE INSULIN RECEPTOR IN ARTIFICIAL LIPID MEMBRANES

Utilizing a system of affinity-purified human insulin receptors reconstituted in intact, unilamellar phospholipid vesicles we have evaluated the role of membrane thickness, lipid saturation, and cholesterol content in the binding and signal transduction of the insulin receptor. In unsaturated phosphatidylcholines, increasing fatty acyl chain length lowers the affinity of the receptor for insulin, and this is associated with a parallel decrease in insulin-stimulated autophosphorylation. These properties of the receptor, however, are dissociated by reconstitution of receptors into vesicles of saturated phosphatidylcholines. Thus, when receptors are inserted into liposomes composed of phosphatidylcholines with saturated fatty acids of chain length from C_8 to C_{20}, insulin binding remains intact up to a chain length of C_{18}, whereas above C_{14} chain length there is a marked decrease in insulin-stimulated receptor autophosphorylation (Fig. 4). The coupling between binding and kinase activation can be restored in part by use of fatty acids with increasing degrees of unsaturation. These results are consistent with a model in which interactions between the receptor transmembrane domain and the hydrophobic membrane environment regulate binding and signal transduction by the insulin receptor. Modification of membrane phospholipids by enzymatic treatment of the cell also modifies receptor signaling (Zoppini and Kahn, 1992).

F. *In Vitro* MUTANTS OF THE INSULIN RECEPTOR TRANSMEMBRANE DOMAIN

As an alternative approach to exploring the role of the transmembrane (TM) domain in signal transduction, we have recently prepared a series of *in vitro* mutants of the receptor in which the sequence of the TM domain

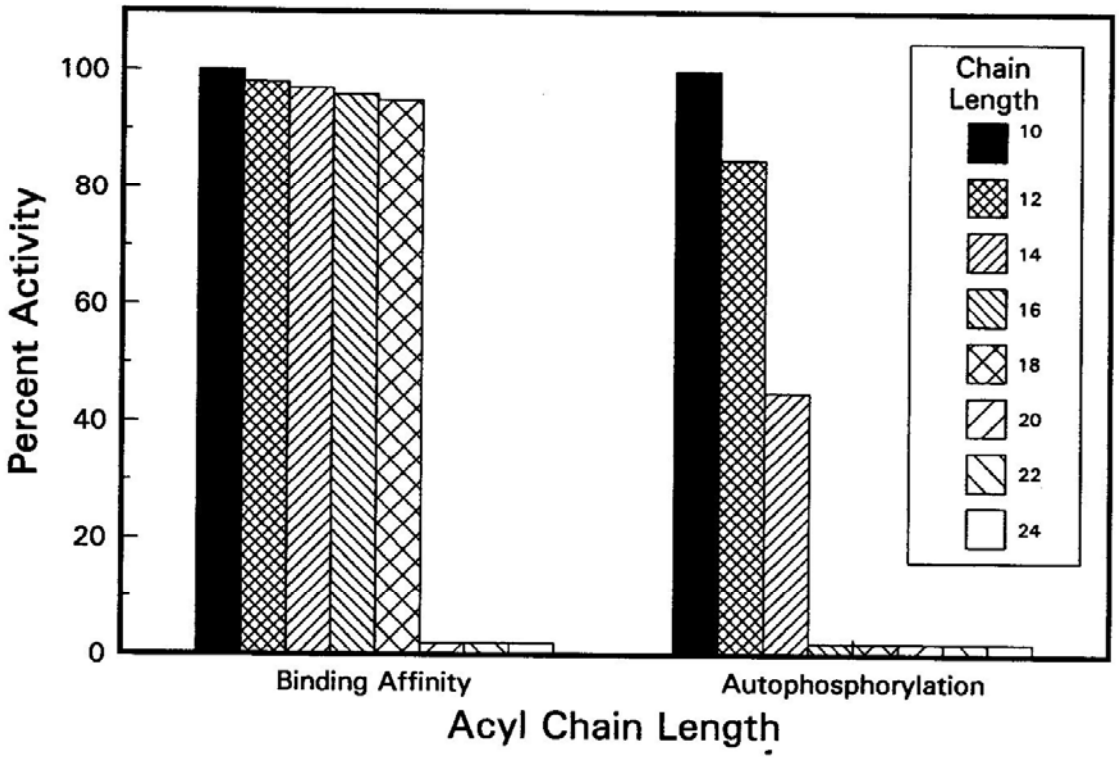

FIG. 4. Effect of membrane lipids on insulin receptor function—activity in unsaturated phospatidylcholine liposomes. Purified placental insulin receptors were reconstituted in unilamellar liposomes consisting of unsaturated phosphatidylcholines of different fatty acyl chain lengths, varying from C_{10} to C_{24}. Insulin binding and receptor autophosphorylation were then assessed using standard techniques.

has been modified in a variety of ways. These include substitution of other receptor TM domains into the insulin receptor, increasing TM length, point modifications of specific TM residues, and flipping the orientation of the TM domain with or without the associated charged residues that flank this domain on its exofacial and cytoplasmic surface. As has been observed with other receptor systems, the TM domain of the insulin receptor can be modified significantly with no apparent change in receptor properties. Substitution of the PDGF receptor TM domain, adding three amino acids to or deleting five amino acids from the TM domain, has no effect on binding or kinase properties (Frattali *et al.*, 1991; Yamada *et al.*, 1992). However, some changes in the TM domain do affect receptor function, indicating several important functional aspects of the TM domain.

One interesting series of mutants that we have produced is one in which the TM domain has a substitution of Val-938 with Asp, substitution of the entire TM domain with that of the c-*neu* protooncogene, or substitution of the TM with that from the *neu* oncogenic homologue (the c-*neu* TM domain containing a point mutation). This series is of interest because in

the c-*neu* protooncogene (a relative of the EGF receptor), the tyrosine kinase activity of the protein is normally very low. Modification of a single Val in the c-*neu* TM domain to the acidic residue Glu results in the constitutive activation of the tyrosine kinase and cellular transformation (Bargmann *et al.*, 1986). In the insulin receptor, a Val-to-Glu or Val-to-Asp point mutation alone has no effect on the receptor kinase activity (Frattali *et al.*, 1991; Yamada *et al.*, 1992); however, substitution of the complete c-*neu* TM domain in the insulin receptor will produce partial activation of the kinase *in vitro* (Fig. 5) (Yamada *et al.*, 1992). Very recently we found that substitution of the *neu* Val → Glu TM domain produces an even greater, and perhaps fully constitutive, activation of the insulin receptor kinase *in vivo* (B. Cheatham *et al.*, submitted for publication). The mechanism of this activation is uncertain, but in the case of the *neu* oncoprotein, activation of the kinase has been ascribed to a tendency of the protein to self-aggregation (Weiner *et al.*, 1989).

Other mutations in the insulin receptor TM may also alter the function of the receptor. For example, almost all transmembrane proteins have one basic amino acid on the extracellular face and three on the intracellular face, which presumably serve as sequences that define the position of the

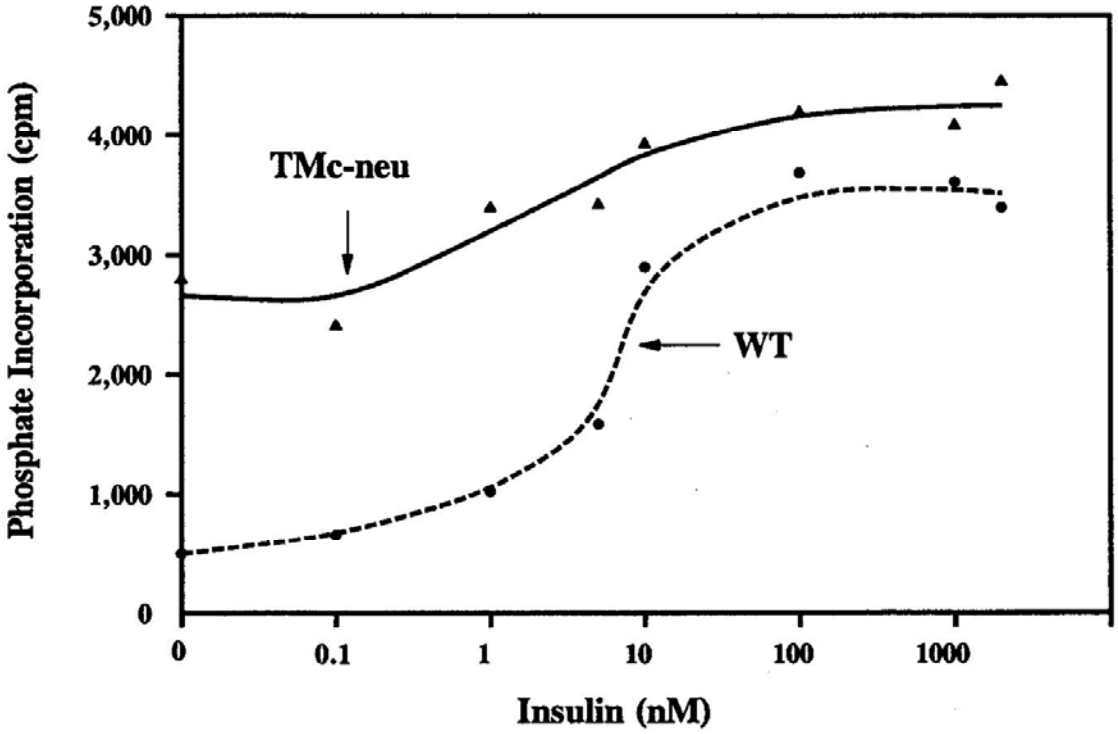

FIG. 5. *In vitro* autophosphorylation of insulin receptors with a transmembrane substitution of the c-*neu* TM for the normal (WT) insulin receptor TM sequence. Note the basal, i.e., constitutive, activation of the kinase. Adapted from Yamada *et al.* (1992).

protein into the membrane. If an insulin receptor is constructed with a TM containing the same amino acids in reversed direction, and if the intracellular flanking charged amino acids are also moved to the extracellular face, the resultant receptor has markedly altered intracellular trafficking, with over 80% remaining in the golgi or endoplasmic reticulum of the cell (K. Yamada *et al.*, in preparation). The 10–20% of receptor that reaches the plasma membrane is normally processed and binds insulin normally, but fails to transmit a normal signal for kinase activation in response to insulin binding.

The TM domain of most tyrosine kinase receptors is viewed as a single α-helical stretch of 21 to 24 amino acids (Yarden and Ullrich, 1988). The insulin receptor TM is also predominantly α-helical, but interestingly contains a Gly and Pro sequence, which should act as "helix breakers," and this feature may also contribute to the function of the receptor. Thus, when the insulin receptor TM is mutated to convert the Gly-Pro in the normal sequence to Ala-Ala, amino acids that should promote increased helicity, there is a threefold increase in mobility in the lipid matrix of the membrane as assessed by fluorescent photobleaching and a similarly increased rate of insulin-stimulated internalization (Goncalves *et al.*, submitted). This is associated with a two- to threefold increase in cell-associated insulin degradation and an increased rate of receptor down-regulation. Thus, nature seems to have provided a "kink" in the insulin receptor TM domain to slow its mobility in the membrane and slow the rate of internalization and degradation of both ligand and receptor.

G. THE INTRACELLULAR REGION OF THE β SUBUNIT

The intracellular or cytoplasmic region of the β subunit consists of 402 amino acids, which can be functionally divided into four domains: a juxtamembrane domain important in substrate binding and receptor internalization, a kinase catalytic domain, a region of tyrosine autophosphorylation involved in regulation of kinase activity, and a C-terminal domain containing two additional tyrosine phosphorylation sites, as well as some sites of serine and threonine phosphorylation (Fig. 6). This structure was in large partially predicted prior to actual cloning of the receptor molecule based on extensive characterization of its tyrosine kinase properties (Kasuga *et al.*, 1982b; White *et al.*, 1984; Avruch, 1989).

The most easily identified domain of the β subunit was the catalytic domain that contains the ATP binding site. In contrast to the extracellular and transmembrane domains, this region contains the highest degree of homology between the insulin receptor and all other members of the protein tyrosine kinase family (Yarden and Ullrich, 1988; White and Kahn,

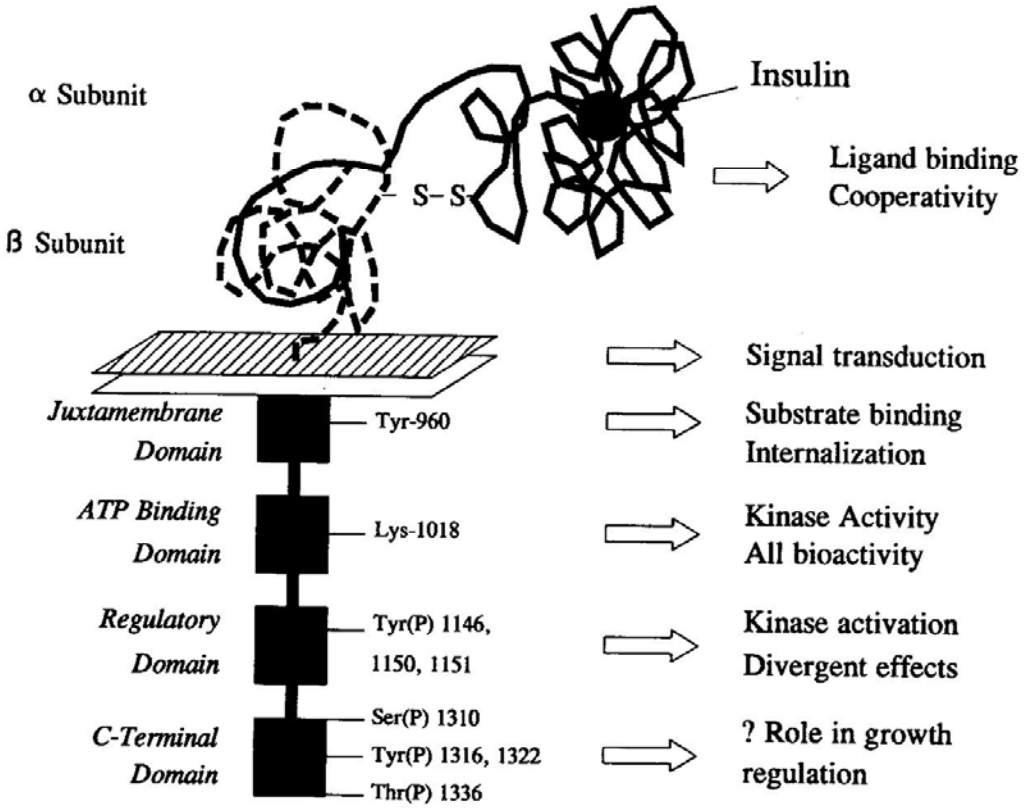

FIG. 6. A detailed view of the insulin receptor β subunit and its intracellular domains. The numbering system used for the amino acid residues refers to the − exon 11 sequence. For the + exon 11 sequence, add 12 to each.

1986). The distinctive motif Gly-X-Gly-X-X-Gly (where X is any amino acid) followed by a lysine residue 10 to 20 amino acids toward the C terminus forms the consensus amino acid sequence encoding the ATP binding domain. The lysine residue (number 1018 in the − exon 11 receptor and 1030 in the + exon 11 receptor) is absolutely required for kinase activity; substitution of this residue with any other amino acid invariably blocks autophosphorylation and kinase activity and most, if not all, biological response mediated by tyrosine kinase receptors (Chou *et al.*, 1987; Ebina *et al.*, 1987). A patient with the type A syndrome of insulin resistance and acanthosis nigricans has been described in which the Gly-X-Gly-X-X-Gly motif is modified by mutation of the final Gly to Val (Yamamoto-Honda *et al.*, 1990). This results in a kinase inactive receptor and a syndrome of severe insulin resistance.

About 110 amino acids toward the C terminus of the β subunit is a cluster of three tyrosine residues (1146, 1150, and 1151 in the − exon 11 receptor and 1158, 1162, and 1163 in the + exon 11 variant) that form a major site of autophosphorylation and constitute the regulatory region of

the kinase. As in other kinases, these tyrosine residues are near acidic amino acids (glutamic or aspartic acid) that increase their affinity as substrates of the receptor kinase. In the insulin receptor, autophosphorylation occurs on all three of these tyrosine residues within seconds after insulin stimulation (White *et al.*, 1988b; Tornqvist and Avruch, 1988; Tornqvist *et al.*, 1988; Tavare *et al.*, 1988; Rosen, 1987), and this increases the activity of the kinase toward exogenous substrates dramatically (Fig. 7). As long as these three residues remain tyrosine phosphorylated, the kinase remains activated, even if insulin is removed from the insulin binding site. Not surprisingly, these three tyrosines also form the preferential site of dephosphorylation of the receptor by cellular phosphotyrosine phosphatases (King and Sale, 1990).

A second major region of phosphorylation of the insulin receptor is the C-terminal domain. This domain contains two tyrosine residues (1316, 1322, or 1328 and 1334 in the two receptor variants), both of which are autophosphorylated by the insulin receptor (White *et al.*, 1988b). In addition, this C-terminal domain contains at least one threonine (1336/1348) and one serine (either 1293 or 1294 in the − exon 11 variant or 1305/1306 in the + exon 11 variant) site of receptor phosphorylation by exogenous Ser/Thr protein kinases (Lewis *et al.*, 1990a,b). The exact role of these phosphorylations in insulin action remains debated. The tyrosine kinase activity of C-terminally truncated receptors is normal (Goren *et al.*, 1987; Maegawa *et al.*, 1988; Myers *et al.*, 1991). Serine/threonine phosphorylation, on the other hand, may play a kinase regulatory role (see below). Several groups have studied receptors bearing C-terminal deletion mutations for other actions with varying results. In our hands, an *in vitro* mutant of the receptor with a deletion of the terminal 34 amino acids, including all of the proposed tyrosine, serine, and threonine sites of phosphorylation, exhibits a normal biological activity profile (Myers *et al.*, 1991). By contrast, Thies *et al.* (1989) have reported that a similar mutant is defective in mediating metabolic effects of insulin while being superactive in stimulating growth effects. The reason for these very discordant results is not clear.

The fourth domain of the β subunit of distinct function is the intracellular juxtamembrane region. Although we initially had proposed that this region was free of tyrosine sites of autophosphorylation (White *et al.*, 1988a,b), more recent data from our laboratory and others (Tornqvist *et al.*, 1988; Tavare *et al.*, 1988) indicate that there are one or more sites of tyrosine phosphorylation in this domain as well. These sites of autophosphorylation, however, are not required for kinase activation, and account for less than 20% of the tyrosine phosphate in the β subunit following insulin stimulation.

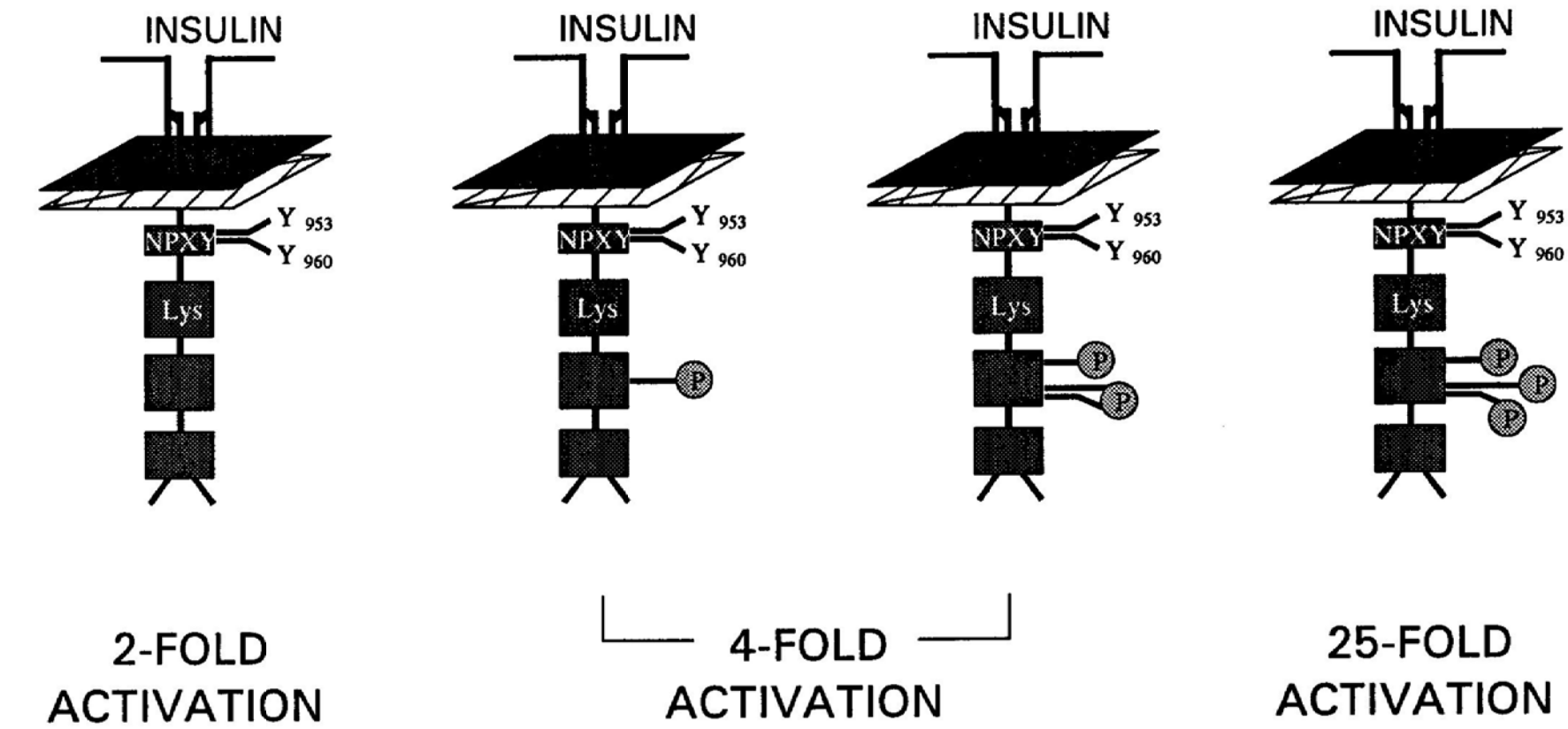

FIG. 7. (A) Schematic model of activation of the insulin receptor tyrosine kinase by the autophosphorylation cascade. (B) Behavior of the kinase toward exogenous (excess) substrates after activation by autophosphorylation. The dotted line represents the activity of the unactivated (basal) receptor; the dashed line represents the activity of the partially activated receptor; the solid line represents the activity of the fully activated (trisphosphorylated) receptor.

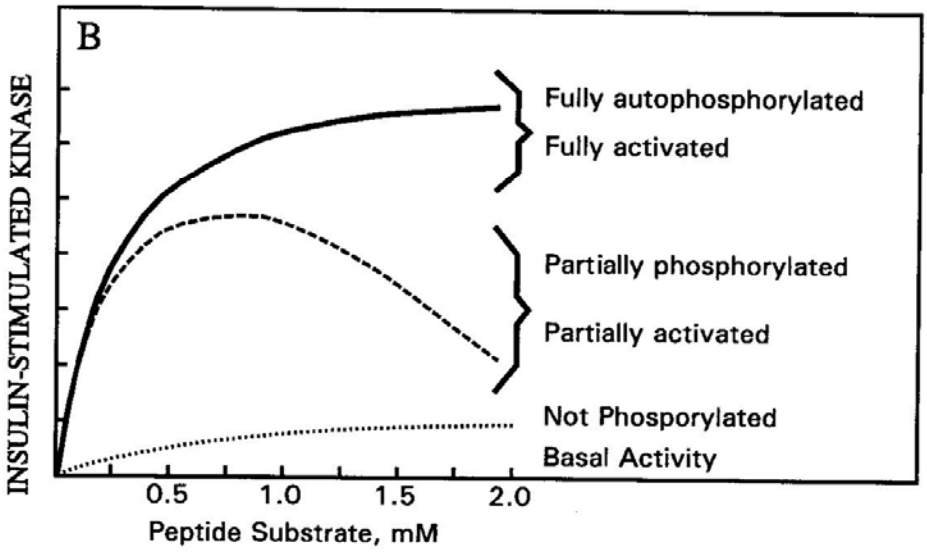

FIG. 7.—*Continued*

The juxtamembrane domain, however, appears to play an important role in several other functions of the receptor. Thus, mutation of the tyrosine at position 960 to phenylalanine (White *et al.*, 1988a), or deletion of a 12-amino acid peptide from this region (Rothenberg *et al.*, 1991), results in a receptor that is kinase active, but fails to phosphorylate the endogenous substrate of the insulin receptor pp185/IRS-1 (see below). Deletion mutations of this region also interfere with insulin-stimulated receptor internalization (Backer *et al.*, 1990; McClain *et al.*, 1987; Thies *et al.*, 1990). This latter effect has been ascribed to the fact that this region of the receptor contains a consensus sequence for internalization Asn-Pro-X-Tyr (NPXY), which is also present in a number of other receptors that exhibit high internalization rates (Chen *et al.*, 1990). This domain also contributes to an extended region of the kinase domain. As a result, the deletion mutant in the juxtamembrane region exhibits an altered K_m for ATP (Backer *et al.*, 1991).

H. THE INSULIN RECEPTOR AS AN ALLOSTERIC ENZYME

Although the exact molecular changes that result in activation of the insulin receptor following insulin binding are uncertain, considerable data suggest that the insulin receptor behaves as a classic allosteric enzyme undergoing both conformational changes and modification by phosphorylation. Following insulin binding, the receptor undergoes a propagated conformation change. This conformational change results in alterations in

the receptor's sensitivity to proteolysis (Donner and Yonkers, 1983; Lipson *et al.*, 1986), susceptibility to chemical modification (Schenker and Kohanski, 1988; Wilden and Pessin, 1987; Waugh and Pilch, 1989), behavior on gel chromatography (Ginsberg *et al.*, 1976), and sedimentation properties (Florke *et al.*, 1990). This conformational change is propagated through both subunits and is associated with changes in antibody recognition to domains within the α and β subunits (Perlman *et al.*, 1989; Baron *et al.*, 1990; Prigent *et al.*, 1990; Herrera and Rosen, 1986; Herrera *et al.*, 1985). In the intact cell, this conformational change is also associated with increased receptor aggregation (Kahn *et al.*, 1981).

Under normal circumstances, the major function of the α subunit is to suppress the kinase activity in the β subunit. Removal of all or part of the α subunit by either limited proteolysis (Shoelson *et al.*, 1988) or *in vitro* mutagenesis (Ellis *et al.*, 1988; Villalba *et al.*, 1989) results in activation of the receptor in a manner similar to that induced by insulin binding. In the intact receptor, the insulin induced conformational and/or aggregational changes somehow reduce the normal effect of the α subunit to suppress the kinase activity in the β subunit. As a result, there is an increase in the kinase catalytic activity. This in turn results in autophosphorylation of the receptor on multiple tyrosine residues, initially involving the cluster of three regulatory tyrosines, as well as the juxtamembrane tyrosines, and the C-terminal tyrosines. And this in turn activates the kinase toward exogenous substrates.

It is still uncertain whether the autophosphorylation reaction is a cis event, i.e., occurs within a single β subunit, or a trans event between β subunits. Based on experiments with tryptically produced isolated receptor halves, we have shown that a cis-phosphorylation reaction can definitely occur, but trans phosphorylation is kinetically favored (Shoelson *et al.*, 1988). On the other hand, autophosphorylation of the isolated intracellular domain of the β subunit suggests a concentration-dependent intermolecular (i.e., trans) reaction (Cobb *et al.*, 1989). Pessin and colleagues have recently presented evidence for an even more complex form of trans reaction in which the two kinase domains in the receptor tetramer phosphorylate each other in a "ping-pong" fashion (Treadway *et al.*, 1991). In IGF-1/insulin receptor hybrids, IGF-1 will induce autophosphorylation of both the insulin and IGF-1 receptor β subunits (Moxham *et al.*, 1989). In either case, phosphorylation of the three regulatory tyrosines produces a further conformational change and activates the receptor kinase toward other cellular substrates, such as the endogenous substrate pp185/IRS-1 described below. Thus begins the chain of events that ultimately culminates in insulin action.

I. REGULATION OF THE RECEPTOR MULTISITE PHOSPHORYLATION

Like many allosteric enzymes, the insulin receptor undergoes multisite phosphorylation, which contributes to its overall regulation. The most important component of this multisite phosphorylation is the trisphosphorylation of tyrosines 1158, 1162, and 1163 (1146, 1150, and 1151 in the − exon 11 variant), which activates the kinase toward exogenous substrates. The importance of these phosphorylations in insulin action is illustrated by a series of seven *in vitro* mutants that we have prepared; in each of these, tyrosines have been mutated singly, doubly, or all together to phenylalanine, and then expressed stably in Chinese hamster ovary cells. Modification of even a single tyrosine to phenylalanine in this regulatory domain results in about a 50% decrease in receptor autophosphorylation and kinase activity (Fig. 8). Mutation of two tyrosines to phenylalanine produces a 70% reduction in kinase activity and mutation of all three tyrosines to phenylalanine causes an almost complete loss of kinase activity to a level similar to that seen in the ATP binding site mutant A1030 (Wilden *et al.*, 1992b). A similar change is also observed if the three tyrosines are changed to three serines, even though the serines can serve as potential phosphate acceptors. These changes in autokinase activity are paralleled by a similar decrease in phosphorylation of the endogenous substrate pp185/IRS-1 (see below).

These mutations produce alterations in downstream biological responses to insulin, but the exact extent to which these responses appear to be coupled varies to a considerable degree (Table II). For example, the ability of insulin to stimulate glucose incorporation into glycogen (a metabolic response) and thymidine incorporation into DNA (a growth response) tends to decrease in proportion to the decrease in receptor autophosphorylation and kinase activity (Wilden *et al.*, 1992a). By contrast, even a single tyrosine-to-phenylalanine mutation in the regulatory region causes a complete loss of insulin-stimulated receptor internalization; thus this insulin effect seems to be most tightly coupled to a normal kinase and autophosphorylation cascade. At the other extreme is insulin-stimulated receptor serine and threonine phosphorylation. This effect is mediated via cellular kinases distinct from the receptor, although the specific mechanism is unknown (Lewis *et al.*, 1990b). Although this insulin-stimulated effect is lost in the ATP binding site mutant (A1030), it is observed normally even in cells containing mutations of all three regulatory tyrosines. Thus, insulin-induced receptor serine phosphorylation is very insensitive to any change in the tyrosine kinase activity of the receptor or receptor autophosphorylation.

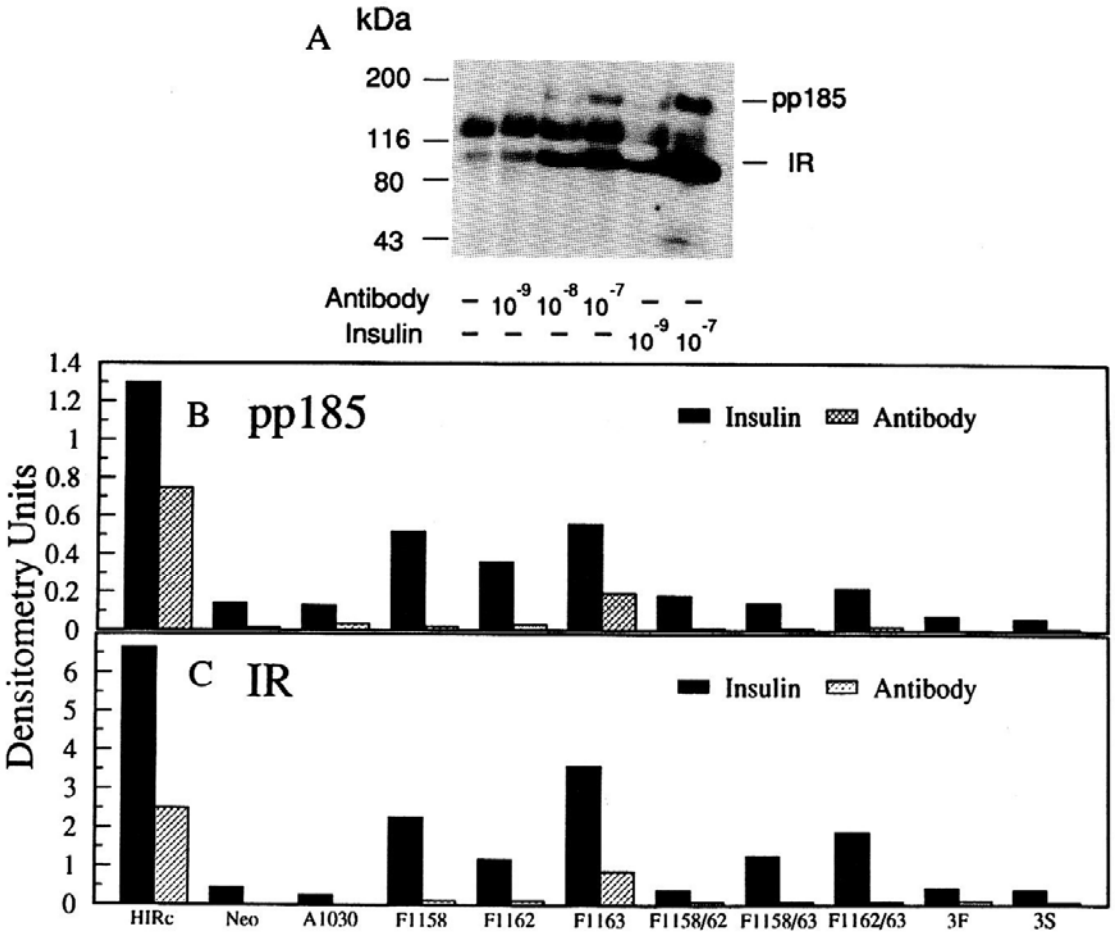

FIG. 8. Activity of the insulin receptor and a variety of mutants following insulin and antibody stimulation. CHO cells transfected with the insulin receptor or mutants were stimulated with insulin or antireceptor antibody 83-14 at a concentration of 100 n*M;* cells were extracted and subjected to SDS–gel electrophoresis and blotting with antiphosphotyrosine antibody. (A) A typical autoradiogram of insulin and antibody stimulation in cells expressing the wild-type human receptor. (B and C) These data are quantitated and compared with results of cells expressing mutant receptors or control vectors. Abbreviations: HIRc, normal human insulin receptor; Neo, neomycin control vector; A1030, ATP binding site mutant; and F1158, F1162, and F1163, receptors with changes in tyrosine to phenylalanine at residues 1158, 1162, and 1163 (the three regulatory tyrosines). The numbering system used in this figure represents that of the + exon 11 variant receptor.

Work in our laboratory and others has suggested that there may be a differential loss of some bioactivities in different single and double tyrosine mutants. For example, mutation of Tyr-1158 to phenylalanine resulted in a receptor that tends to lose growth-promoting activity more than metabolic activity (Wilden *et al.*, 1990). Debant *et al.* (1989) have reported that mutation of the other two regulatory tyrosines (1162 and 1163) tends to result in a preferential loss of metabolic activity over growth-promoting action. Neither of these findings has been reproducible in all studies (Zhang

TABLE II
Summary of Insulin Receptor Mutant Function[a]

Receptor	Serine phosphorylation	Kinase active	pp185 phosphorylation	DNA synthesis	Glycogen synthase	Internalization
Wild type	+	+	+	+	+	+
C terminal deletion	+	+	+	+	+	+
Juxtamembrane	+	+	−	−	−	−
Regulatory 1-Phe	+	<	<	<	<	−
Regulatory 2-Phe	+	<<	<<	<<	<<	−
Regulatory 3-Phe	+	−	−	−	−	−
Kinase deficient	−	−	−	−	−	−

[a] The activities studied include insulin stimulation of receptor serine phosphorylation, kinase activity, pp185 phosphorylation, DNA synthesis, glycogen synthesis, and receptor internalization. The receptor mutants include the C-terminal deletion; the juxtamembrane (F960) mutant; mutants of 1, 2, or 3 tyrosines to phenylalanine in the regulatory region, and the kinase-deficient (ATP binding site) mutant.

et al., 1991; Wilden *et al.*, 1992a). Whether the small differences in biological responses observed in *in vitro* mutants are significant is not clear, but similar differential biological response losses have been observed in some natural mutants of the insulin receptor that have been reported in syndromes of insulin resistance (Moller *et al.*, 1990a,b).

In contrast to the activation of the receptor kinase produced by tyrosine autophosphorylation, insulin also stimulates the serine and threonine phosphorylation of the insulin receptor (Kasuga *et al.*, 1982b). Indeed, in intact cells this accounts for more than half of the total phosphate incorporated into the receptor on prolonged insulin stimulation. In general, the stimulation of tyrosine phosphorylation is very rapid (maximal within 15 seconds), whereas the stimulation of serine/threonine phosphorylation is more gradual and occurs over several minutes to 1 hour (White *et al.*, 1984). In cell-free systems, serine/threonine phosphorylation of the receptor is observed with protein kinase C and to a variable degree with cAMP protein kinase (Bollage *et al.*, 1986; Tanti *et al.*, 1987). In intact cells, serine phosphorylation is stimulated by phorbol esters and cAMP analogs, suggesting that these may be the kinases physiologically involved in this process (Takayama *et al.*, 1988; Stadtmauer and Rosen, 1986; Häring *et al.*, 1986). An insulin receptor-associated serine kinase has also been observed in partially purified receptor preparations, which appears to be distinct from these kinases (Czech *et al.*, 1988; Lewis *et al.*, 1990b). Thus far, however, this insulin-stimulated receptor serine kinase (IRSK) has not been isolated. The physiological importance of serine/threonine phosphorylation is uncertain. Receptors isolated from phorbol ester-treated cells or receptors phosphorylated *in vitro* by protein kinase C exhibit decreased tyrosine

kinase activity and some decreased biological responses (Takayama *et al.*, 1988). Thus, it appears that the insulin receptor can be positively regulated by tyrosine phosphorylation and negatively regulated by serine phosphorylation, producing a highly controlled kinase activity.

II. Substrates of the Insulin Receptor

Transmission of an insulin signal following activation of the receptor kinase could potentially involve several different pathways. In the most direct model of signal transmission, the activated kinase would catalyze the phosphorylation of an endogenous substrate or substrates (Fig. 9). Initial attempts to identify such substrates relied primarily on reconstitution experiments in which tyrosine phosphorylation of potential substrates was assessed by incubation of the partially purified receptor with candidate proteins *in vitro*. Under these conditions, the receptor will phosphorylate a large number of proteins, including histones, calmodulin, and lipocortins; however, the physiological significance of these events is impossible to ascertain (Rosen, 1987). Alternatively, attempts have been made to identify substrates by SDS–gel electrophoresis using insulin-stimulated ^{32}P-labeled cells and phosphoamino acid analysis. Using this approach, some relatively abundant proteins that have a low level of insulin-induced tyrosine phosphorylation may be identified; however, this approach is gener-

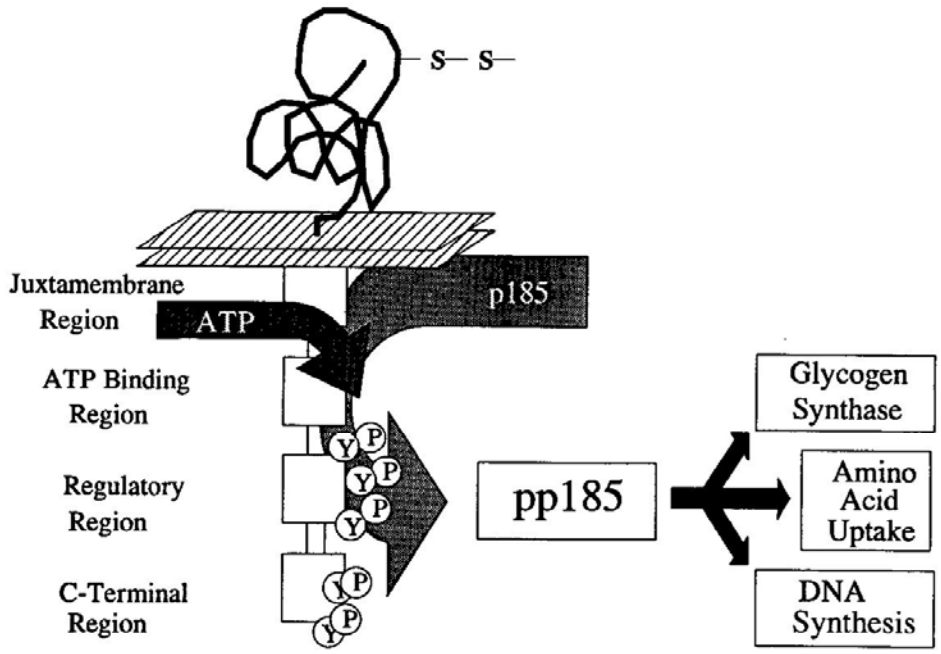

FIG. 9. A simple substrate model of the insulin receptor β subunit and a single substrate leading to signaling in all pathways.

ally both cumbersome and insensitive. A major step toward identifying the important physiological substrates of the insulin receptor was the development of antibodies to phosphotyrosine (Pang *et al.*, 1985). These antibodies can then be used to immunoprecipitate the P-Tyr-containing proteins of ^{32}P-labeled cell extracts or immunoblot proteins from cell extracts before and after insulin stimulation. Because cells also contain many phosphotyrosine phosphatases, it is important to include a variety of inhibitors of these enzymes.

An example of a study using antiphosphotyrosine antibodies to study insulin-induced tyrosine phosphorylation is shown in Fig. 10. In this study, extracts of normal rat liver were made before and after insulin injection into the portal vein; the extracts were then subjected to SDS–gel electrophoresis, transferred to nitrocellulose paper, and immunoblotted with an antiphosphotyrosine antibody. In the absence of insulin stimulation, normal rat liver contains only two significant phosphotyrosine-containing proteins: one has an apparent molecular mass of 120 kDa and the other has a molecular mass of about 175 kDa. The 120-kDa protein (pp120) is observed as the major phosphotyrosine-containing protein of most cell

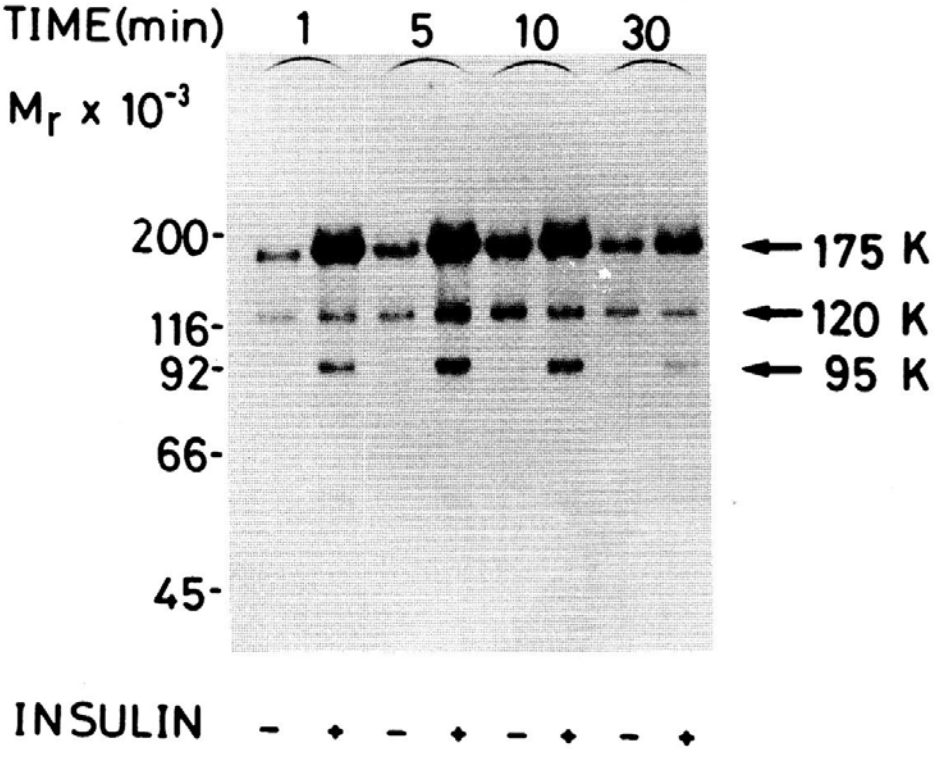

FIG. 10. Time course of insulin stimulation of receptor and substrate phosphorylation in normal rat liver. Rats were injected in the portal vein and at various times their livers were extracted, SDS gels were performed, and the resulting blots were reacted with an antiphosphotyrosine antibody.

types. Recent work in our laboratory and that of Schaller *et al.* (1992) suggested that this protein corresponds to an endogenous tyrosine kinase associated with focal adhesion placques. The 175-kDa protein corresponds to the EGF receptor and can be precipitated and blotted with anti-EGF receptor antibody.

Following insulin stimulation, there is a rapid appearance of a two new phosphotyrosine-containing bands. One corresponds to the 95-kDa β subunit of the insulin receptor; the other is a broad band between 165 and 185 kDa that overlaps the EGF receptor and is strongly insulin stimulated. However, based on a number of criteria, including antibody recognition, this higher molecular mass band is not the EGF receptor, but instead represents the major endogenous substrate of the insulin receptor. We initially termed this protein pp185 based on its electrophoretic mobility (White *et al.*, 1985); more recently we have renamed it insulin receptor substrate-1, or IRS-1, because its actual molecular mass is much less than the observed 185 kDa (Sun *et al.*, 1991).

In addition to pp185/IRS-1, adipocytes contain at least two other proteins, pp60 and pp46, which show some degree of insulin-stimulated tyrosine phosphorylation (Rothenberg *et al.*, 1991). Also, a protein of 15 kDa can be observed to undergo tyrosine phosphorylation in 3T3-L1 cells following insulin stimulation if the cells are first treated with phenylarsene oxide (Hresko *et al.*, 1988). This protein has been subsequently identified as the abundant fatty acid binding protein (AP2 or 422) (Bernier *et al.*, 1987). In liver, insulin can also be shown to stimulate phosphorylation of endogenous lipocortin 2, but this is best observed after induction of the protein by glucocorticoid administration (Karasik *et al.*, 1988). Interestingly, very few other proteins that undergo insulin-stimulated tyrosine phosphorylation are observed in cells or tissues using this technique (Rosen, 1987). Because pp185/IRS-1 is the major endogenous substrate in most cell types, it has received the most extensive characterization.

A. INSULIN RECEPTOR SUBSTRATE-1/pp185

Some of the characteristics of pp185/IRS-1 are listed in Table III. As noted above, this protein is observed in virtually all tissues and cell types studied. This includes rat adipocytes (Monomura *et al.*, 1988; Mooney *et al.*, 1989; DelVecchio and Pilch, 1989), rat liver (Rothenberg *et al.*, 1991; Tobe *et al.*, 1990), cultured rat hepatoma cell lines (White *et al.*, 1985, 1987; Witters *et al.*, 1988; Tornqvist *et al.*, 1988; Tashiro-Hashimoto *et al.*, 1989), rat myoblasts (Beguinot *et al.*, 1989; Burdett *et al.*, 1990), mouse 3T3-L1 adipocytes (Gibbs *et al.*, 1986; Madoff *et al.*, 1988; Witters *et al.*, 1988), mouse fibroblasts (Pasquale *et al.*, 1988; Brindle *et al.*, 1990), mouse

TABLE III
Characteristics of Insulin Receptor Substrate-1 (IRS-1/pp185)

The major substrate of insulin in most cells
Is rapidly phosphorylated and dephosphorylated on tyrosine residues in response to insulin stimulation
Phosphorylation correlates with insulin action in cells expressing mutant insulin receptors
Coprecipitates with insulin-stimulated PI 3-kinase activity
Phosphorylated on serine and threonine in basal state and on tyrosine after insulin stimulation
Phosphorylation in regulated in physiologic and pathologic states

neuroblastoma cells (Shemer *et al.*, 1987), Chinese hamster ovary cells (White *et al.*, 1987; Chou *et al.*, 1987), human epidermoid carcinoma cells (Kadowaki *et al.*, 1987) and human adipocytes (Thies *et al.*, 1990). The kinetics of phosphorylation are rapid and reversible. In cultured cells pp185 phosphorylation is near maximal within 15–30 seconds after insulin stimulation (White *et al.*, 1985), and the time course is similar in intact tissues following intraportal injection of insulin (Rothenberg *et al.*, 1991) (Fig. 10). When insulin is removed from the cell, there is also a rapid dephosphorylation of pp185. Thus, the kinetics of response are consistent with the rapid kinetics required for insulin signaling of cells. On phosphopeptide and phosphoamino acid analysis, pp185 is multiply phosphorylated (White *et al.*, 1985; X.-J. Sun *et al.*, submitted for publication). In the basal state, most, if not all, of the phosphorylation is on serine and threonine; however, following insulin stimulation there is significate phosphorylation on tyrosine residues as well. Stimulation of tyrosine phosphorylation of IRS-1 (identified as pp160 in this tissue) is also observed in a differentiation-dependent manner in 3T3-L1 preadipocytes, consistent with their increasing insulin sensitivity (Keller *et al.*, 1991). IRS-1 phosphorylation is also observed in a number of cell types following incubation with IGF-1 (Beguinot *et al.*, 1989; Madoff *et al.*, 1988; Izumi *et al.*, 1987; Kadowaki *et al.*, 1987; Shemer *et al.*, 1987; Condorelli *et al.*, 1989). In many of these cases IGF-1 is probably acting through its own receptor, which is highly homologous to the insulin receptor. IRS-1 phosphorylation, however, is not observed following stimulation by other growth factors that work through other receptor tyrosine kinases, such as EGF or PDGF.

In addition to these characteristics, studies with cells expressing *in vitro* mutated insulin receptors suggested that pp185 was an important physiological substrate of the receptor. Thus, in cells expressing insulin receptors in which the ATP binding sites of the receptors or the regulatory tyrosines have been mutated, there is a decrease in pp185 phosphorylation that closely parallels the decrease in receptor autophosphorylation and induction of insulin action (Fig. 8) (Wilden *et al.*, 1992b).

The most important evidence that pp185 might be important in insulin action, however, came from studies of the insulin receptor that was mutated in the juxtamembrane region. As noted above, there are multiple tyrosine residues in the insulin receptor β subunit that may undergo autophosphorylation, including at least two (Tyr-953 and Tyr-960) in the region just inside the membrane of the cell. In an effort to determine the role of these tyrosines in signal transduction, two mutant receptors were made, one in which Tyr-960 was converted to phenylalanine (IR-F960) and the other in which a 12-amino acid region, including Tyr-953 and Tyr-960 was deleted (IR-Δ960). When these were expressed in cells, they were found to have normal kinase activity and to undergo insulin-stimulated tyrosine autophosphorylation in a normal or very near-normal manner (Fig. 11, left). Despite this, these two mutant receptors were unable to phosphorylate pp185 on tyrosine residues (Fig. 11, right) (White *et al.*, 1988b). This was not because of an absence of pp185/IRS-1 in these cells, because the protein could be observed on immunoblots using anti-IRS-1 antibodies. Most importantly, when insulin action was studied, these mutant insulin receptors were found to be inactive in supporting insulin-stimulated biological effects, including activation of glycogen synthase, stimulation of

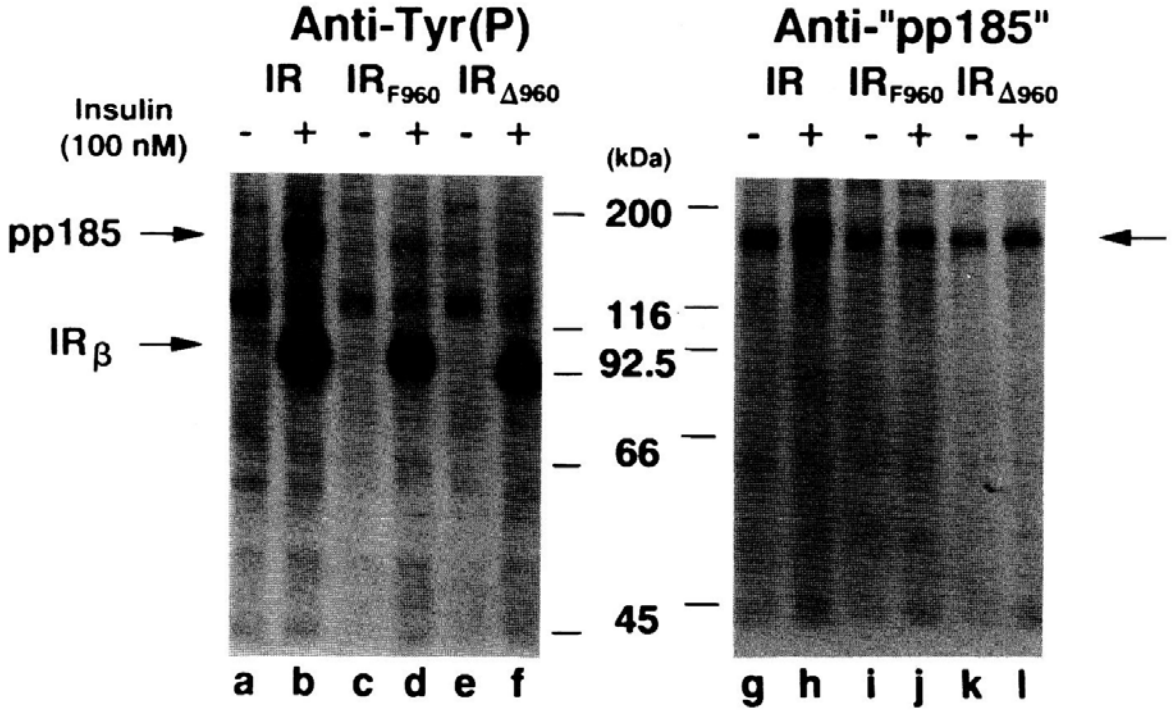

FIG. 11. Phosphorylation of the insulin receptor (pp185) and its substrate in CHO cells expressing normal and mutant insulin receptors in the juxtamembrane domain after ^{32}P labeling. Proteins were immunoprecipitated with antiphosphotyrosine or anti-pp185 antibodies.

amino acid uptake, and thymidine incorporation into DNA (Fig. 12). Thus, receptors that are kinase active but cannot phosphorylate pp185 fail to transmit an insulin signal to more distal events. Hence, IRS-1/pp185 appears to play a critical role in the action of insulin.

Based on these findings, our laboratory undertook the task of purification of this protein. This was very difficult and challenging for several

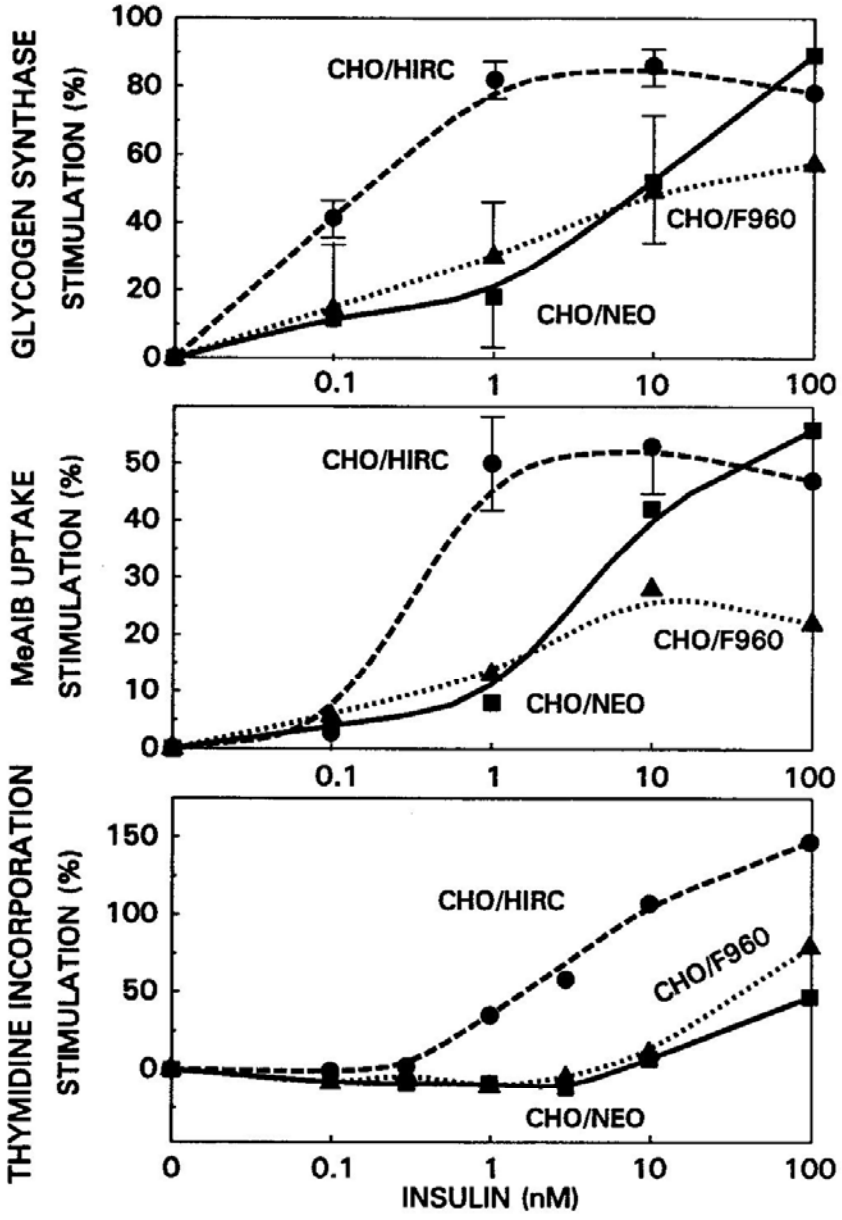

FIG. 12. Biological effects in cells expressing the normal insulin receptor (HIRC) or the Phe-960 (F960) mutant and control cells transfected with the neomycin (NEO) vector only. Top: Insulin stimulation of glycogen synthase. Middle: Insulin stimulation of (methyl-AIB) amino acid uptake. Bottom: Thymidine incorporation into DNA. Note that in every case the normal insulin receptor enhances insulin sensitivity whereas the F960 mutant does not.

reasons. First, pp185 is a very nonabundant protein in cells (about the same order of abundance as the insulin receptor), and no cell types were observed with very high levels of expression. Second, the only tools available initially for purification were antiphosphotyrosine antibodies, and pp185 was known to be very easily and rapidly tyrosine dephosphorylated even in the presence of high concentrations of inhibitors of PTPases. Finally, rat liver (the tissue chosen for purification), contains another protein of similar molecular weight and isoelectric point, carbamyl phosphate synthase (CPS), which is 100 to 1000 times more abundant than pp185. Thus, even using antiphosphotyrosine affinity columns to purify cell extracts, there was always significant contamination of the preparation with CPS.

To deal with these problems, a strategy was developed in which 50–100 rats were injected in the portal vein with insulin (a similar number of controls were injected with saline), the livers were excised at 1 minute and frozen in liquid nitrogen, the proteins were extracted in buffers containing high levels of PTPase inhibitors, the extracts were purified on anti-P-Tyr antibody columns and analyzed by SDS–gel electrophoresis, and the protein obtained in both the basal and insulin-stimulated states were subjected to tryptic peptide mapping by reverse-phase HPLC. Over 140 peptides were obtained from the tryptic digest of the pp185 band from the basal and stimulated samples, and 10 of these were new or increased following insulin injection (Rothenberg *et al.*, 1991). Each of these 10 was then subjected to microsequencing. In most cases, two amino acids were identified at each cycle. The more abundant one represented an amino acid from a peptide of CPS that contaminated the preparation; the less abundant amino acid represented a new peptide that was assumed to be from IRS-1. These amino acid sequences could then be used to make antipeptide antibodies and to predict the sequence of the mRNA, which should code IRS-1.

Using oligonucleotides based on this sequence information, two rat liver cDNA libraries were screened and ultimately sufficient clones were obtained to derive a full-length sequence for IRS-1 (Sun *et al.*, 1991). The complete IRS-1 cDNA was 5.4 kb in length and contained a single open reading frame of 3.4 kb. Northern blot analysis revealed that IRS-1 has a single dominant mRNA of ~9.5 kb. This coded for a hydrophilic protein of 1235 amino acids with a predicted molecular mass of 131 kDa. The difference between the predicted size of the protein and the apparent size on SDS gels is due to two factors. First, the protein exhibits anomalous migration. Following *in vitro* translation and SDS–gel electrophoresis, the apparent M_r = 160,000. In addition, the migration is further retarded due to phosphorylation on serine and threonine residues, as well as on tyrosine

residues.This migration property explains the varied molecular weight estimates in studies from different laboratories. In view of the discrepancy between the true molecular mass and the apparent size on SDS gels, we now favor the name insulin receptor substrate-1, or IRS-1, for this protein. Recently we cloned the human muscle IRS-1 molecule (Araki *et al.*, submitted for publication) and find this protein to be very highly conserved (88% at the amino acid level) across species and tissues.

IRS-1 is shown schematically in Fig. 13 and appears to represent a new class of signal transduction molecules (Sun *et al.*, 1991). Consistent with its cytoplasmic location, IRS-1 is a hydrophilic molecule and contains no stretches of hydrophobic amino acids sufficiently long to transverse the membrane of the cell. Chou–Fasman analysis suggests that it has less than 5% helical content and is composed primarily of β sheets, β turns, and random-coil domains. Compared to a typical globular protein with 30–70% α helix, IRS-1 has much higher β-sheet content. Inspection of the sequence reveals a number of potential functionally important motifs. Near the N terminus of the molecule is a potential ATP (or GTP) binding site, beginning with a glycine-rich motif (Gly-X-Gly-X-X-Gly) and followed by an essential lysine residue 14 amino acids away in the sequence Ala-X-Lys. However, IRS-1 lacks the typical Asp-Phe-Gly and Ala-Pro-Glu motifs diagnostic of a protein kinase. There is also another potential nucleotide binding site near the C terminus in rat liver IRS-1, but this site is not fully conserved in the human muscle IRS-1 molecule, suggesting that it is less likely to be functionally important (Araki *et al.*, submitted).

IRS-1 contains many potential phosphorylation sites. Based on the typical motifs for cyclic AMP-dependent protein kinase, protein kinase C, casein kinase II, and cdc2 kinase, rat liver IRS-1 contains a total of 35 potential sites of serine or threonine phosphorylation distributed throughout the molecule (Fig. 13). Due to the slight differences in amino acid sequence, in human IRS-1 there are even more potential Ser/Thr phosphorylation sites (>50). Although it is not yet known which of these sites are phosphorylated in the intact cell, IRS-1 is highly serine and threonine phosphorylated, and this phosphorylation is increased following treatment of cells with phorbol esters, suggesting that IRS-1 is a substrate of protein kinase C (White *et al.*, 1985).

The most striking features of the molecule are the potential tyrosine phosphorylation sites, at least 10 of which can be identified based on the presence of tyrosine residues close to the acidic amino acids, glutamic or aspartic acid. Six of these are in a repetitive motif Tyr-Met-X-Met (YMXM), three have the sequence Tyr-X-X-Met (YXXM), and one has the sequence Glu-Tyr-Tyr-Glu (EYYE). For several of the YMXM sites there appears to be an even larger type of consensus motif in which this

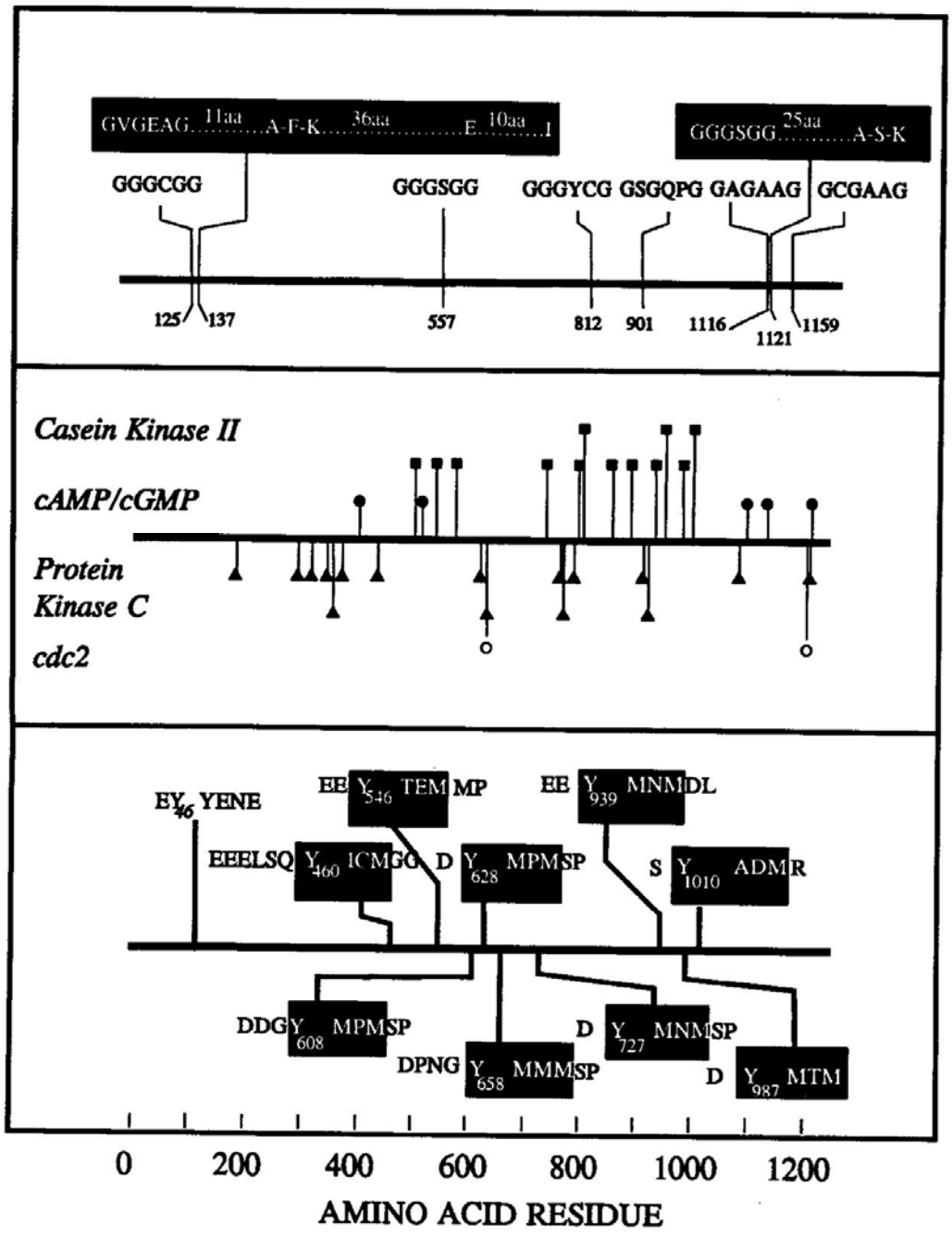

FIG. 13. Schematic representation of some of the structural features of IRS-1. Top: Potential nucleotide binding sites. Middle: Potential sites of serine or threonine phosphorylation. Bottom: Potential sites of tyrosine phosphorylation.

sequence is preceded by a Gly-Asp and followed by a Ser-Pro (SP). Thus, a longer overall consensus GDYMXMSP motif exists. According to the predicted secondary structure, all of the YMXM and YXXM motifs exist in a β sheet. Synthetic peptides containing these YMXM motifs are excellent high-affinity substrates of the insulin receptor kinase *in vitro*, with K_m

values in the low micromolar range (Shoelson *et al.*, 1992). Substitution of the methionine residues with other amino acids reduces the substrate efficiency of these peptides, suggesting that the YMXM motif is a key determinant for substrate recognition. The occurrence of this type of repetitive motif suggests some type of gene duplication event; however, a specific pattern consistent with this type of event (if this occurred) is not apparent at the nucleotide level. All 10 of these potential tyrosine phosphorylation sites are conserved exactly in the human IRS-1 molecule. Interestingly, several potential Ser/Thr phosphorylation sites exist very close to our overlapping YMXM or YXXM motifs, but the significance of this relationship is unknown at present.

Comparison of the IRS-1 sequence with sequences in the gene data banks reveals no similar proteins. The YMXM tyrosine phosphorylation motifs, however, are observed in several other tyrosine kinases and tyrosine kinase-related proteins, including two in the kinase intervening sequence of the PDGF receptor and one each in the EGF receptor, polyoma middle T protein, c-KIT, c-FMS, and several other oncogene tyrosine kinases (Table IV). In addition, several tyrosine kinases, including the insulin receptor itself, have a YXXM type sequence.

Recent studies have suggested that YMXM (and perhaps YXXM) phosphorylation motifs serve a special function in signal transduction by acting as intracellular recognition sites for other signaling proteins. In this model, when the YMXM motif is phosphorylated on the tyrosine (Y) residue, the

TABLE IV
Proteins with YMXM Motifs

Protein	Tyr	Motif
IRS-1	608	SNLHTDDGYMPMSPGVAPV
Middle T antigen	298	TQAERENEYMPMAPQIHLY
PDGF-A Receptor	731,742	GDYMDMKNADTTQYVPML E
PDGF-B Receptor	740,751	GGYMDMSLDESVDYVPML D
EGF Receptor	920	PPICTIDVYMIMVKCWMID
C-KIT	718	PSCDSSNEYMDMKPGVSYV
C-FMS	721	FSSQGVDTYVEMRPVSTSS
cAMP-Dependent K	118	SFKDNSNLYMVMEYVAGGE
Insulin Receptor	1322	RSYEEHIPYTHMNGGKKNG
IRS-1	546	SSVVSIEEYTEMMPAAYPP

affinity of this domain for a specific recognition domain in other signaling molecules is increased (Fig. 14). This $Y^{(P)}MXM$ recognition domain has been termed an SH2 domain because it was first recognized as a domain homologous to a region of the *src* oncogene product (Sugimoto *et al.*, 1984; Whitman *et al.*, 1985, 1987). Many oncogene tyrosine kinases and several normal intracellular enzymes contain SH2 domains (Table V). The SH2 domain contains enzymes, including a phosphatidylinositol 3-kinase (Carpenter *et al.*, 1990; Shibasaki *et al.*, 1991; Escobedo *et al.*, 1991; Skolnik *et al.*, 1991; Otsu *et al.*, 1991), phospholipase C_γ (Margolis *et al.*, 1990), the G protein activating protein GAP (Anderson *et al.*, 1990; Kazlauskas *et al.*, 1990), and at least one phosphotyrosine phosphatase (Shen *et al.*, 1991). When the phosphorylated YMXM domain binds the SH2 domain of these enzymes, the enzymes may be regulated in their activity or changed in their subcellular localization. Thus, IRS-1 may be viewed as a "docking protein" with multiple YMXM motifs, several of which are phosphorylated and couple this protein to downstream enzymes involved in the insulin action cascade (Fig. 14).

There are two implications of this model. The first is that phosphorylation of IRS-1 results in an association of this protein with one or more molecules containing SH2 domains and this somehow changes their relative activity in the cell. Second, one might predict that it would be possible to mimic or block this interaction using isolated YMXM phosphopeptides. Thus far the only enzymatic activity definitely shown to associate with IRS-1 is the phosphatidylinositol 3-kinase (PI 3-kinase). Indeed, when insulin binds to its receptor and activates the receptor kinase, resulting in IRS-1 phosphorylation, there is an association of the regulatory (85 kDa)

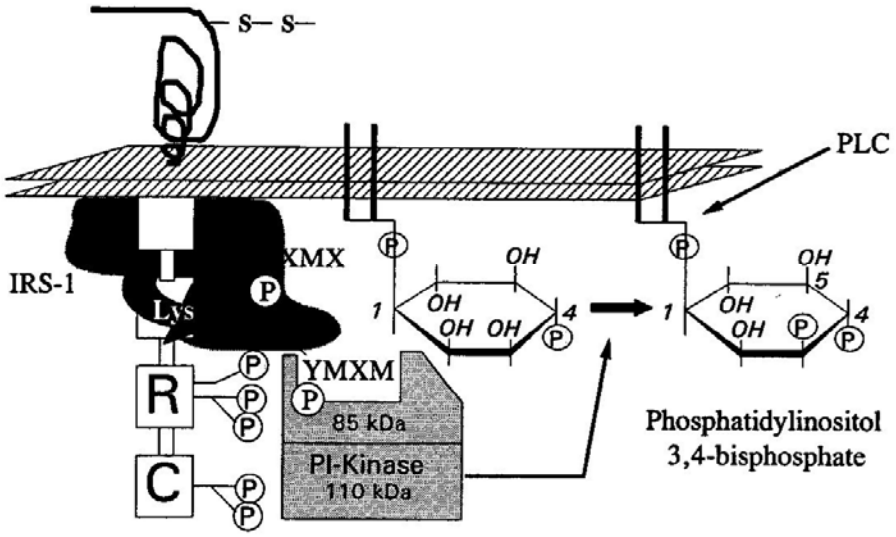

FIG. 14. Model showing role of IRS-1 and PI 3-kinase in insulin action.

TABLE V
Cellular Ligands and SH2 Domains

SH2 domain ligands	SH2 domain proteins
	Tyrosine kinases
PDGF receptor	v-fps
EGF receptor	v-src
Polyoma middle-T antigen	v-crk
CSF-1 receptor	v-fgr
pp185/IRS-1	v-abl
	v-yes
	PLC_γ
	PI 3'-kinase
	G protein activating protein (GAP)
	Phosphotyrosine phosphatase(s)

subunit of the PI 3-kinase with IRS-1, which can be detected by immunoblotting and a 5- to 10-fold stimulation of the IRS-1-associated PI 3-kinase activity (Fig. 15) (Ruderman *et al.*, 1990; Backer *et al.*, 1991, 1992; Kapeler *et al.*, 1991). This results in phosphorylation of phosphatidylinositol (PI), PI 4-phosphate, and PI 4,5-bisphosphate on the 3-position to form PI-3-P, PI-3,4P_2, and PI-3,4,5-P_3 (Ruderman *et al.*, 1990; Endemann *et al.*, 1990).

A similar model has been derived for the interaction of other tyrosine kinases and their substrates with SH2-containing molecules (Anderson *et al.*, 1990; Koch *et al.*, 1991). The exact role of PI-3-P and related compounds in cellular metabolism is not yet known. However, PI 3-kinase is also activated by several other growth factor receptors (Cantley *et al.*, 1991), and this activation can be observed *in vivo* at physiological concentrations of insulin (Folli *et al.*, in press), suggesting that this reaction may play a role in the growth-promoting actions of these peptide hormones and growth factors. Cells expressing mutant insulin receptors that fail to phosphorylate IRS-1, such as the F960 and Δ960 mutants, do not activate the PI 3-kinase and do not transmit a downstream insulin signal (Kapeler *et al.*, 1991; Backer *et al.*, 1992).

It is still unclear exactly how tyrosine phosphorylation of IRS-1 is linked to other insulin actions. A theoretical model is shown in Fig. 16 (also see below). In addition to IRS-1 and PI 3-kinase, Maasen and colleagues have shown that insulin stimulates GTP loading of *ras* (Burgering *et al.*, 1989, 1991), and GAP (which forms complexes with *ras*) is known to contain SH2 domains (Anderson *et al.*, 1990; Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990). Thus far, however, we have been unable to find any association between the insulin receptor or IRS-1 and the *ras*–GAP complex (B.

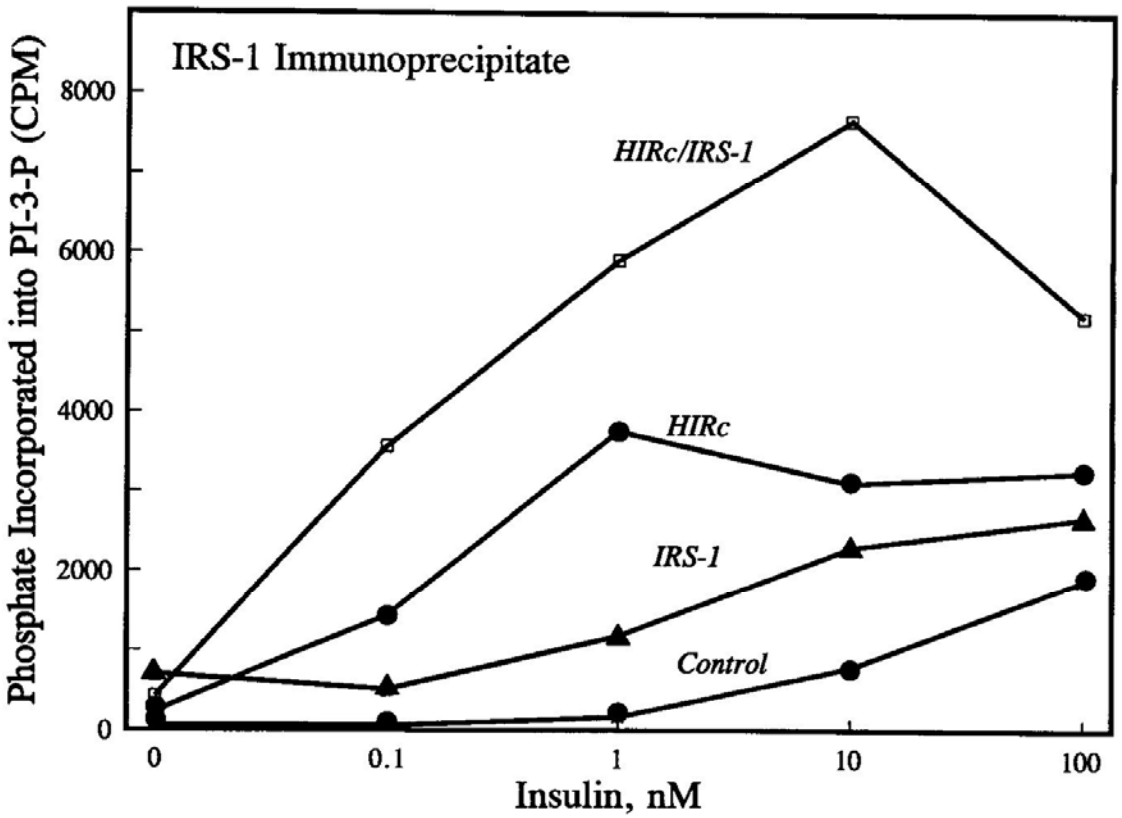

FIG. 15. Insulin stimulation of PI 3-kinase in CHO cells transfected with the human insulin receptor (HIRc), rat IRS-1 (IRS-1), both (HIRc/IRS-1), or neither (control). In each case the PI 3-kinase activity was measured in IRS-1 immunoprecipitates after insulin stimulation of the intact cells.

Cheatham *et al.*, unpublished data). Also there is no evidence of association of phospholipase C_γ (Meisenhelder *et al.*, 1989) or a phosphotyrosine phosphatase (Shen *et al.*, 1991) with IRS-1, although thus far studies along this line have been relatively limited.

Because the insulin action cascade ultimately involves a number of serine/threonine phosphorylation events, it has been suggested that one of the molecules that might associate with IRS-1 via SH2 domains would be a serine/threonine kinase, which could act as a "switch kinase," converting the signal system from a tyrosine to serine phosphorylation cascade (Czech, 1989). Mitogen-activated protein (MAP) kinase is a candidate for such an intermediate because this enzyme appears to be central to the action of a number of growth factors and is activated by insulin (Ray and Sturgill, 1988; Rossomando *et al.*, 1989). MAP kinase requires both tyrosine and threonine phosphorylation for activity, a property expected for a switch kinase (Blenis, 1991). In the cytoplasm, MAP kinase has been shown to play a role in activation of the ribosomal S6 protein kinase (RSK)

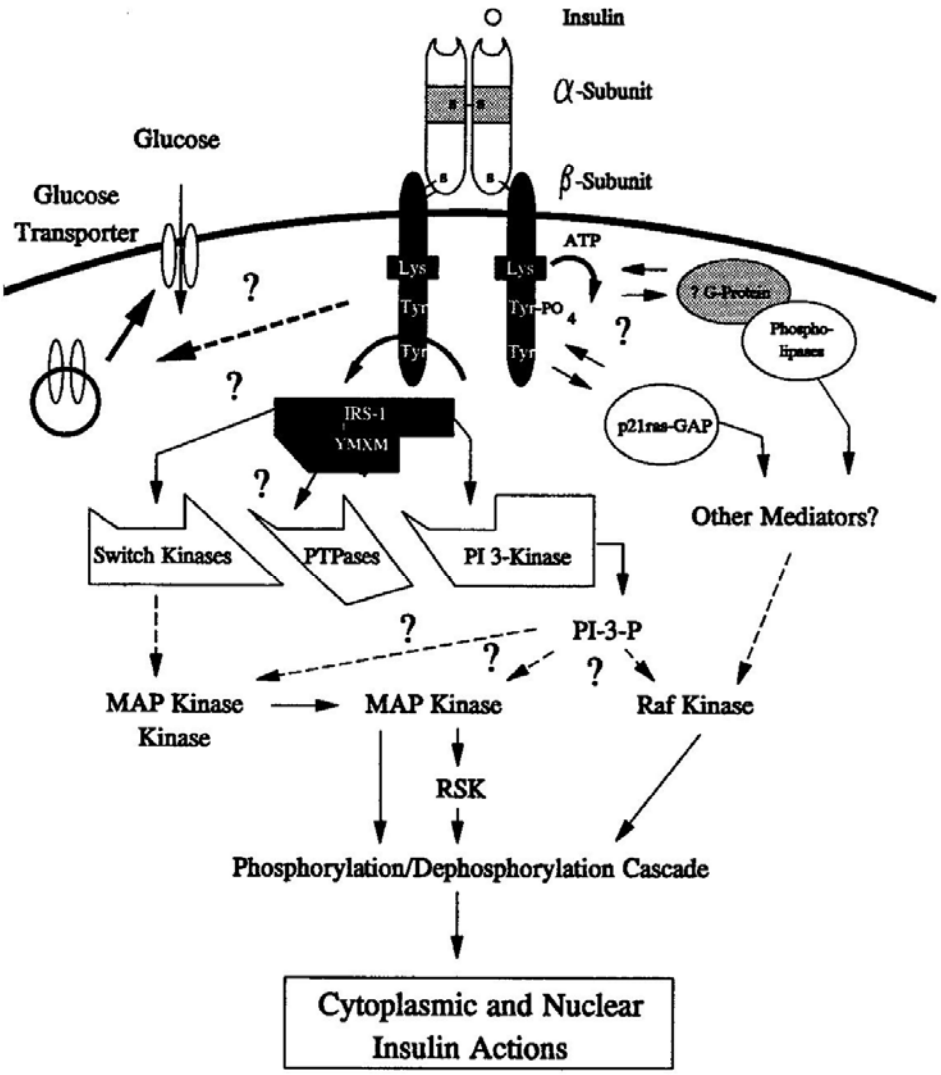

FIG. 16. A more complex (and possibly complete) model of the molecular events involved in early steps of insulin action.

kinase, another component of the insulin action cascade (Rosen, 1987). Furthermore, MAP kinase can translocate between the cytoplasm and nucleus and could mediate some of the insulin actions at both levels. We have shown that insulin stimulation can affect protein serine phosphorylation at both the cytoplasmic and nuclear level (Csermely and Kahn, in press).

The *raf*-1 kinase, which has been shown to be capable of both tyrosine and serine/threonine phosphorylation and is a substrate for other tyrosine kinases, would also be candidate for this type of intermediate (Li *et al.*, 1991). Indeed, insulin has been shown to stimulate the phosphorylation of *raf* kinase; however, this is on serine residues and is not due to an IRS-1 or insulin receptor association (Kovacina *et al.*, 1990; Blackshear *et al.*, 1990). Thus, it seems likely that at least one additional step in the cascade

of early events in insulin action needs to be defined to complete the linkage between insulin-stimulated tyrosine and serine/threonine phosphorylations.

B. IS TYROSINE PHOSPHORYLATION INVOLVED IN INSULIN ACTION?

Although the insulin receptor is clearly insulin-stimulated tyrosine kinase and considerable evidence suggests that the kinase activity is required for insulin action, the exact role of the kinase activity in insulin action continues to be debated. Evidence for the role of the receptor kinase activity in insulin action is substantial and includes the following points:

1. The insulin receptor is an insulin-stimulated tyrosine protein kinase, and thus far, no other activities have been directly associated with the receptor.
2. Overexpression of kinase-active insulin receptors in cells increases the sensitivity of the cell to all measurable insulin-stimulated actions, whereas overexpression of kinase-inactive insulin receptors has no effect (Ebina *et al.*, 1987; Chou *et al.*, 1987).
3. Both spontaneously occurring natural receptor mutants (Taylor *et al.*, 1990b) and *in vitro*-produced receptor mutants with a defective ATP binding site or altered sites of autophosphorylation (Wilden *et al.*, 1992a) are ineffective in transmitting a normal insulin signal when expressed in cells.
4. Injection of cells with antibodies to the insulin receptor β subunit (Morgan and Roth, 1987) or to phosphotyrosine inhibits the actions of insulin. Likewise, microinjection of *Xenopus* oocytes with a phosphotyrosine phosphatase will inhibit insulin action (Cicirelli *et al.*, 1990).

There are several reported findings, however, that indicate that insulin action may involve more than simple kinase activation. First, although mechanistically unexplained, several investigators have reported insulin-stimulated responses in cells overexpressing kinase-inactive receptors (Gottschalk, 1991) or receptors in which all three regulatory tyrosines have been mutated (Sung *et al.*, 1989; Rafaeloff *et al.*, 1991). This includes insulin stimulation of pyruvate dehydrogenase, S6 kinase, and even amino acid uptake. What is confusing is that many of these same effects have been found to be defective when studied by other investigators using similar receptor constructs (Chou *et al.*, 1987; Wilden *et al.*, 1992b). Whether this indicates that similar insulin actions in different cells utilize different pathways or that some methodological differences account for the varied results has not yet been determined. Perhaps only a small

amount of kinase activation is needed to imitate postreceptor events, and the differences lie in the sensitivity of the assays used to study the kinase activation.

A second finding has been used to suggest that kinase activity is not required for insulin action, i.e., that antiinsulin receptor antibodies are capable of mimicking insulin action without stimulating the receptor kinase (Forsayeth *et al.*, 1987) or can mimic insulin actions in cells expressing phosphorylation-defective receptors that fail to respond to insulin (Debant *et al.*, 1989). In our experience, as well as in the work of others, neither of these facts is uniformly reproducible. In many studies antiinsulin receptor antibodies do stimulate the insulin receptor kinase (Brindle *et al.*, 1990; Wilden *et al.*, 1992b; Steele-Perkins and Roth, 1990), although they are slightly less potent than insulin on a molar basis (Fig. 15). Also, we have failed to see any effect of antireceptor antibody to stimulate a classical insulin action, such as glycogen synthesis or DNA synthesis, in cells expressing mutant receptors that fail to respond to insulin (Wilden *et al.*, 1992a). The reasons for these discrepancies in the data have not been resolved.

C. ALTERNATIVE MECHANISMS OF INSULIN RECEPTOR SIGNALING

Although there is good evidence that insulin action involves stimulation of the receptor tyrosine kinase activity, several additional or alternative mechanisms of action in which the receptor may interact with other signaling molecules covalently or noncovalently may also participate in the full spectrum of insulin mediated events (Fig. 16). As noted above, there is good evidence that the phosphorylated receptor undergoes a conformational change and this change could result in noncovalent interactions of the receptor with other signaling molecules. Several studies have suggested a noncovalent interaction between the insulin receptor and some class of G proteins with insulin-modulating toxin-induced ADP ribosylation (Houslay *et al.*, 1989; Rothenberg and Kahn, 1988; Pyne *et al.*, 1989). Furthermore, toxins that catalyze ADP ribosylation and inactive G proteins block some of insulin's actions (Luttrell *et al.*, 1988; Ciaraldi and Maisel, 1989; Moises and Heidenreich, 1990).

In addition, several investigators have suggested that insulin action may stimulate a specific phospholipase C that is involved in the generation of one or more low-molecular-weight mediators of insulin action (Low and Saltiel, 1988; Saltiel, 1990). In this scheme, insulin stimulates a phosphatidylinositol glycan-specific phospholipase C (perhaps via a G protein),

which would hydrolyze a precursor in the membrane of the cell to produce a PI glycan and 1,2-diacylglycerol, both of which could have regulatory effects on intracellular enzymes. Such mediators have been proposed to play a role in insulin stimulation of pyruvate dehydrogenase, cAMP phosphodiesterase, adenylate cyclase, and phospholipid methyltransferase. Diacylglycerol, a potent activator of protein kinase C, has also been proposed to play a role as an insulin second messenger (Farese and Cooper, 1989). Although support for an important role for any of these alternative mechanisms in insulin action is limited, as noted above studies have suggested that cells expressing mutant insulin receptors with markedly reduced kinase activity may be active for signal transduction, at least along some insulin action pathways. Most likely, insulin signaling involves activation of several pathways. However, we are still rather far from a full understanding of this very complex scheme, and indeed still do not know even where in these action pathways the various components of insulin signaling diverge.

III. Regulation of Early Steps of Insulin Action in Physiologic and Pathologic States

Very soon after methods became available for the study of the insulin receptor, it became clear that insulin receptors were regulated in a variety of physiologic and pathologic states. In fact, at least three previous presentations at The Laurentian Hormone Conference have dealt in some detail with these regulatory events (Roth *et al.*, 1975; Kahn *et al.*, 1981; Taylor *et al.*, 1990a). These studies have indicated that the insulin receptor can be subjected to genetic mutations and regulated in its expression, turnover, affinity, and tyrosine kinase activity and that these changes may play a role in the pathophysiology of a number of disease states. These studies have also indicated that there can be differential regulation of the binding and kinase functions of the receptor. A detailed review of these studies is beyond the scope of this article, but is the subject of many excellent reviews, in addition to those listed above (Häring and Obermaier-Kusser, 1989; Kahn and Goldstein, 1989; Moller and Flier, 1991). Some of the findings of these studies have been very relevant to increasing our understanding of the molecular aspects of insulin by further elucidating some of the structure/function relationships within the receptor and the potential for regulation of function by both genetic and environmental variables. Recently we have begun to extend these studies to the IRS-1 molecule, which could also serve as a site for physiologic regulation of insulin action.

A. REGULATION OF IRS-1 IN PATHOPHYSIOLOGIC STATES

The basic approach for studies of IRS-1 physiology and pathophysiology thus far is analysis of protein tyrosine phosphorylation in liver and muscle using antiphosphotyrosine and anti-IRS-1 antibodies after injection of insulin into the portal vein of mice or rats. This is similar to the technique used initially to identify IRS-1 in tissues of intact animals and allows an analysis of the function of both the receptor and its substrate in the *in situ* condition.

An example of such a study in fed and fasted rats is shown in Fig. 17. As in the previous studies, the major constitutive phosphotyrosine-containing protein in liver and muscle is the pp120 protein. Following insulin stimulation there is rapid stimulation of tyrosine phosphorylation

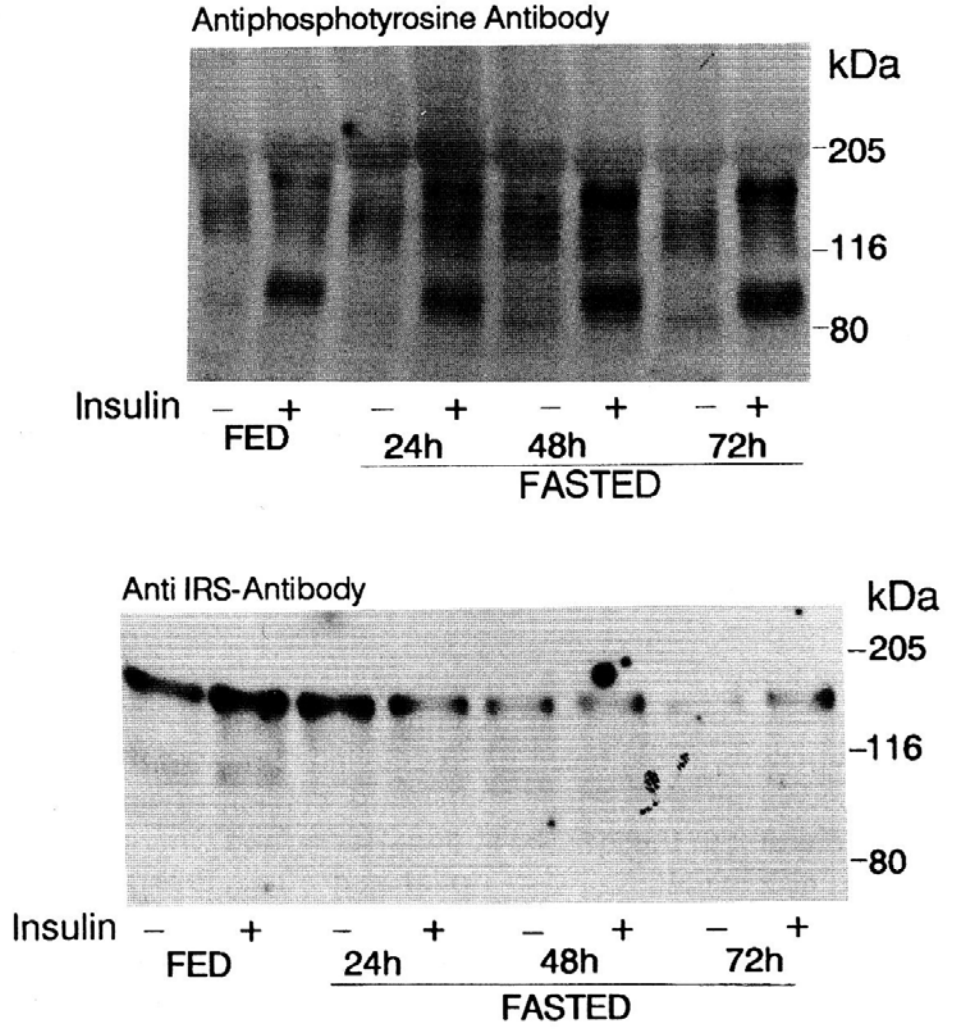

FIG. 17. Insulin receptor and IRS-1 phosphorylation in muscle of fed and fasted rat. The basic technique was as given in Fig. 10; however, in this case skeletal muscle was extracted and immunoblotted with antiphosphotyrosine and anti-IRS-1 antibodies. Adapted from Saad *et al.* (submitted).

of the β subunit of the insulin receptor (95 kDa) and the IRS-1 molecule (pp185). During fasting, there is a progressive increase in the insulin-stimulated phosphorylation of the insulin receptor. This is in part due to the increase in receptor number that occurs as insulin levels fall and receptors are "up-regulated." There is a parallel and even greater increase in the insulin-stimulated phosphorylation of IRS-1. In fact, we had made this observation even prior to beginning the purification of IRS-1, and as a result used fasted rats for the initial purification. Subsequently, when anti-IRS-1 antibodies became available, it became clear that although IRS-1 phosphorylation increased during fasting by almost twofold in liver, this was associated with a *decrease* in the actual amount of IRS-1 protein as determined by immunoblotting (Fig. 17). A similar increase in insulin receptor and IRS-1 phosphorylation is also observed in muscle following fasting, but in this tissue the levels of the IRS-1 protein do not change or may increase slightly.

We have also studied the regulation of IRS-1 in two forms of diabetes: the insulin-deficient diabetes of the streptozotocin (STZ)-treated rat and the insulin-resistant diabetes of the ob/ob mouse. In STZ-diabetic rats there is an increase in insulin receptor number and receptor autophosphorylation. The increase in receptor number again represents up-regulation, which occurs in this hypoinsulinemic state. Despite this increase in receptor number, receptor kinase activity decreases; however, overall there is an increase in receptor autophosphorylation, because the increase in receptor number outweighs the decrease in kinase activity. IRS-1 phosphorylation increases in parallel with the increased availability of the total active receptor kinase pool. As in fasting, IRS-1 protein levels decrease in liver and tend to increase in muscle.

The ob/ob mouse is an obese insulin-resistant rodent that is often used as an animal model of noninsulin-dependent diabetes in humans. In this animal there are very high circulating insulin levels, and down-regulation of the insulin receptor occurs in both liver and muscle. There is also a decrease in receptor kinase activity and overall receptor autophosphorylation is decreased about 50%. In contrast to the findings in fasting and STZ diabetes, in this case there is a significant decrease in IRS-1 phosphorylation, reflecting the overall decrease in receptor kinase activity. At the protein level, the changes are more complex with a decrease in IRS-1 in liver and an increase in IRS-1 in muscle. These changes are summarized in Table VI.

Several conclusions can be derived from these studies. First, IRS-1 phosphorylation is regulated and tends to parallel the changes in insulin receptor phosphorylation, indicating that the enzyme (i.e., the receptor) is rate limiting in the overall reaction in intact cells and tissues. Second,

TABLE VI
Regulation of Insulin Receptor and IRS-1 in Muscle and Liver in Physiologic and Pathologic States[a]

	Insulin Receptor				*IRS-1/pp185*			
	Binding		Phosphorylation		Protein		Phosphorylation	
	Muscle	*Liver*	*Muscle*	*Liver*	*Muscle*	*Liver*	*Muscle*	*Liver*
Fasting	↑↑	↑↑	↑↑	↑	↓↓	↑	↑↑	↑↑
STZ Diabetes	↑↑	↑↑	↑	↑	↓	↑	↑↑	↑
ob/ob Mice	↓↓	↓↓	↓↓	↓	→	↓↓	↓↓	↓↓

[a] Regulation of the insulin receptor and IRS-1 expression and phosphorylation in liver and muscle in various physiologic and pathological states. Adapted from Saad *et al.* (submitted).

the level of IRS-1 protein is regulated, but there are different patterns of regulation in the liver and muscle. In the liver, IRS-1 regulation is similar to insulin receptor regulation, being increased in hypoinsulinemic states and decreased in hyperinsulinemic states. In muscle, the converse is observed with decreased IRS-1 in hypoinsulinemic states and increased IRS-1 in hyperinsulinemic states. Further studies will be needed to determine if and how these changes in IRS-1 protein and phosphorylation play a role in the overall physiology of these disorders.

IV. Conclusions and Future Directions

Over the past 10 years, a great deal of progress has been made in defining many of the steps in insulin action at the cellular and molecular levels. In this review, we have focused our attention on the two earliest molecular events, binding and activation of the insulin receptor kinase and phosphorylation and subsequent signal transduction by the insulin receptor substrate IRS-1. Defining these events has helped add a whole new dimension to our understanding of insulin action. At the same time, studies defining the molecular events at the end of the insulin action cascade, such as activation of glucose transport and regulation of gene expression, have also begun to clarify the specific components required for these signaling events. Although a "black box" remains between the early and late events in insulin action, it is becoming smaller. With further study over the next several years we hope to fill in even more components of this important pathway and ultimately use this knowledge to elucidate the pathophysiology underlying diabetes and to develop new and better therapeutic approaches.

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